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14. ABSTRACT Neurofibromatosis type 2 (NF2) is associated with a homozygous inactivation of the neurofibromatosis 2 (NF2) gene. Despite intense study of the NF2 gene, the mechanism by which the NF2 tumor suppressor acts to prevent tumor formation is not well understood. The goal of this research is to examine the role of posttranscriptional regulation of the NF2 gene. During this reporting period, we have confirmed our findings that vestibular schwannomas express a distinct pattern of alternatively spliced NF2 transcripts lacking specific exons. We have carried out a detailed analysis of NF2 expression during embryonic development and in the tissues affected by NF2 using the transgenic, in situ hybridization and antibody staining approaches. We show that NF2 promoter expression was detected as early as E5.5. Strong NF2 promoter activity was seen in the embryonic ectoderm and in all NF2-affected tissues examined. Importantly, we observed strong NF2 promoter activity in the developing brain and in sites containing migrating cells including the neural tube closure. We also noted a transient change of NF2 promoter activity during neural crest cell migration. While little NF2 promoter expression was detected in premigratory neural crest cells at the dorsal ridge region of the neural fold, significant activity was seen in the neural crest cells already migrating away from the dorsal neural tube. The NF2 promoter expression pattern during embryogenesis suggests a specific regulation of the NF2 gene during neural crest cell migration and further support the role of merlin in cell adhesion, motility, and proliferation during development. By using the conditional gene targeting approach, we have created an <i>Nf2<sup>lox8</sup></i> allele and are in the process of generating a conditional exon 8 knockout mouse. These transgenic and knockout experiments should allow us to study the function of schwannoma-expressed NF2 isoforms in vivo.					
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## INTRODUCTION:

Neurofibromatosis type 2 (NF2) is a hereditary disorder characterized by the development of bilateral vestibular schwannomas (reviewed in Baser et al., 2003; Chang et al., 2005). NF2 is associated with a homozygous inactivation of the neurofibromatosis 2 gene (*NF2*), which encodes a protein named ‘merlin’ for moesin-ezrin-radixin like protein (Traflet et al., 1993). Despite intense study of the *NF2* tumor-suppressor, the mechanism by which merlin acts to prevent tumor formation is not well understood. The *NF2* transcripts undergo alternative splicing, generating a series of mRNA isoforms lacking one or more exons. Presently, the role of alternative splicing of *NF2* mRNAs is not understood. *NF2* isoform 1 (without exon 16) but not isoform 2 (containing all 17 exons) possess growth inhibitory properties (Gutmann et al., 1999). Also, transgenic mice over-expressing the *NF2* isoform with a deletion of exons 2 and 3 in Schwann cell lineage showed a high prevalence of Schwann cell hyperplasia and tumors (Giovannini et al., 1999). These results raise the possibility that functional contribution of the *Nf2* tumor suppressor may require a balanced expression of various isoform proteins in Schwann cells and/or other cell types. In addition, we found that differential usage of multiple polyadenylation sites also contributes to the complexity of human *NF2* transcripts (Chang et al., 2002). Presently, the role of differential polyadenylation of *NF2* transcripts is not known. The goal of the proposed research is to examine the role of posttranscriptional regulation (alternative splicing and differentiation polyadenylation) of the *NF2* gene. Ultimately we hope to provide a better understanding of the mechanisms of NF2 tumorigenesis.

## BODY:

### **Aim 1: Analysis of the Expression Pattern of Alternatively Sliced *NF2* Transcripts in Schwann Cells and Vestibular Schwannomas.**

Task 1: Over the past year, we have procured 10 additional vestibular schwannomas and three paired normal vestibular nerves. RNAs were isolated from these vestibular schwannomas and vestibular nerves and used in the following reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Task 2: Accomplished previously.

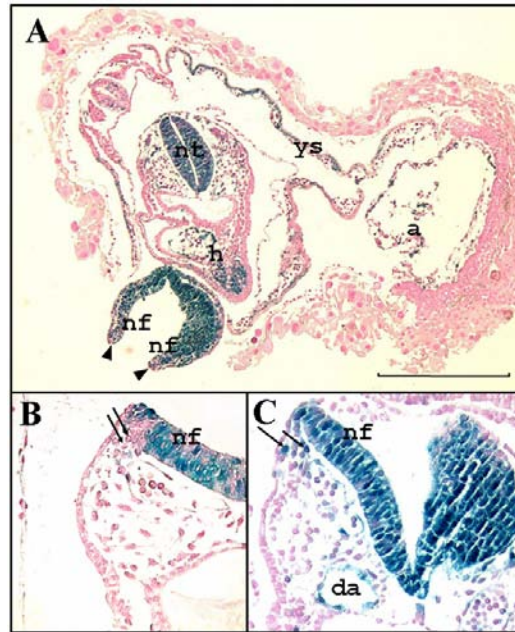
Task 3: By RT-PCR of RNAs isolated from vestibular schwannomas, normal vestibular nerves, and various normal human tissues, we have confirmed our previous findings that the expression pattern and relative frequency of the alternatively spliced *NF2* transcripts in vestibular schwannomas were different from those detected in other human tissues or cell lines. In particular, we found that vestibular schwannomas expressed a high percentage of the *NF2* mRNA isoform lacking exons 15 and 16. We have reported these findings in two reports (one published [Chang et al., 2005] and the other in press [Neff et al., 2005]; see Appendices).

Task 4: We have performed mutation analysis of the *NF2* gene on the DNA isolated from the blood sample from the patient with a sporadic schwannoma that expresses all *NF2* mRNAs lacking exon 8. Sequence analysis revealed that the cDNAs isolated from this schwannoma completely matched the wild-type *NF2* sequence with the exception of the spliced exons. These results suggest that deletion of exon 8 via alternative splicing inactivates *NF2* function in this tumor.

### **Aim 2: Functional Analysis of the Two *NF2* Isoforms Commonly Expressed in Vestibular Schwannomas.**

Task 5: The major progress for this aim is two-fold. First, because we plan to use the authentic *NF2* gene promoter to express the *NF2* isoforms commonly found in vestibular schwannomas in transgenic mice, we carried out a detailed analysis of *NF2* expression during embryonic development and in the tissues affected by neurofibromatosis type 2 using the transgenic, *in situ* hybridization, and antibody staining approaches. We have now completed the analysis and our results showed that the *NF2*

promoter could direct  $\beta$ -galactosidase ( $\beta$ -gal) reporter expression as early as embryonic day 5.5 (E5.5). *NF2* promoter activity was first detected in the embryonic ectoderm and to a lesser extent, in some parts of endoderm and mesoderm. The *NF2* promoter expression pattern in the embryonic tissues was corroborated by *in situ* hybridization analysis of endogenous *Nf2* RNA expression. Importantly, we observed strong *NF2* promoter activity in the developing brain and in sites containing migrating cells including the neural tube closure, branchial arches, dorsal aorta, and paraaortic splanchnopleura. Furthermore, we noted a transient change of *NF2* promoter activity during neural crest cell migration. While little *NF2* promoter expression was detected in premigratory neural crest cells at the dorsal ridge region of the neural fold, significant activity was seen in the neural crest cells already migrating away from the dorsal neural tube (Figure 1). In addition, we detected considerable *NF2* promoter activity in various *NF2*-affected tissues such as acoustic ganglion, trigeminal ganglion, spinal ganglia, optic chiasma, the ependymal cell-containing tela choroidea, and the pigmented epithelium of the retina. The *NF2* promoter expression pattern during embryogenesis suggests a specific regulation of the *NF2* gene during neural crest cell migration and further support the role of merlin in cell adhesion, motility, and proliferation during development. A manuscript describing these findings in detailed has been prepared for publication (see Akhmametyeva et al., 2005 in the Appendices). The *NF2* promoter will be useful for the expression of schwannoma-expressed *NF2* cDNA isoforms in various *NF2*-affected tissues during development.



**Figure 1.** Change of *NF2* promoter activity during neural crest cell migration. (A) A transverse section of the transgenic E8.5 embryo showed significant  $\beta$ -gal expression in neural fold (nf) of the head region, the developing neural tube (nt) and heart (h), as well as yolk sac (ys) and allantois (a). Note that the tip (arrows) of the neural fold displayed weak  $\beta$ -gal staining compared to the rest of the neural fold which exhibited strong  $\beta$ -gal activity. Bar = 100  $\mu$ m. (B,C) Detailed analysis of tissue sections containing the neural fold region revealed that while little  $\beta$ -gal staining was found in the round-shaped neural crest cells (arrows) which were at the moment of delaminating from the dorsal ridge region of the neural fold (B), significant  $\beta$ -gal expression was detected in the neural crest cells already migrating away from the dorsal neural tube (C). da, dorsal aorta.

Second, to examine the effect of expression of the schwannoma-expressed *NF2* isoforms lacking exon 8 *in vivo*, we have also used the embryonic stem (ES) cell-gene targeting strategy to delete the exon 8 of the germline *Nf2* allele. A conditional gene targeting vector containing the exon 8 sequence flanked by two *loxP* recombination sites was generated (Figure 2). The vector also contained a PGK-neo expression cassette for positive selection of recombinant clones. By screening more than 400 G418 (neo)-resistant clones, we have successfully introduced *loxP* recombination sites flanking exon 8 into the endogenous *Nf2* allele. We obtained at least three ES clones that contain a flox allele of *Nf2* (*Nf2*<sup>flox8</sup>).

As shown in Figure 2, Southern blot analysis of EcoRV-digested genomic DNA using the sequence from the exon 7 region as the probe confirmed that clones 1B11, 1G11, and 2A12 contained the 6.5-kb targeted *Nf2* fragment as compared to those with the wild-type allele which yielded a 14.9-kb EcoRV fragment. These targeted ES clones are being injected into blastocysts in order to generate a chimera mouse with the *Nf2<sup>lox8</sup>* allele. Heterozygous and homozygous *Nf2<sup>lox8</sup>* mice will be generated and used to create an exon 8 knockout mouse.

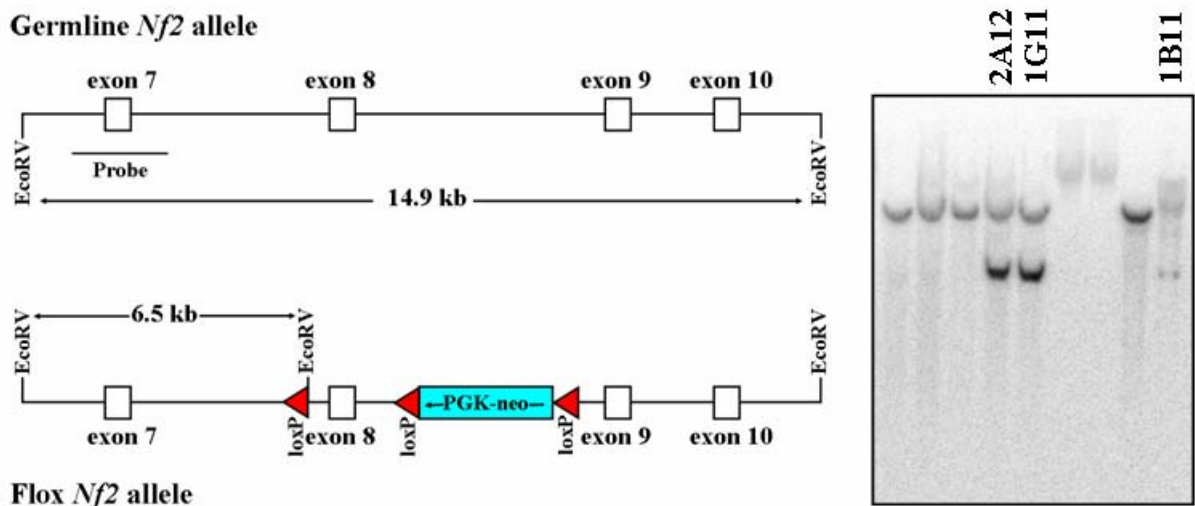


Figure 2. Schematic diagram of the exon 8 targeting construct and identification of ES clones containing a floxed allele of *Nf2* (*Nf2<sup>lox8</sup>*).

Task 6: Accomplished in previous years.

Task 7: Accomplished in previous years. Induced expression of the schwannoma-expressed *NF2* isoforms did not show any effect on the growth properties of 293 kidney and RT4 schwannoma cells.

Task 8: Previously we fused the two schwannoma-expressed *NF2* isoforms with the glutathione-S-transferase (GST) protein. Because we did not observe any biological activities of these schwannoma-expressed *NF2* isoforms in 293 kidney and RT4 schwannoma cells, we are presently focusing on their *in vivo* activities using the approaches described in task 5. It is likely that the transgenic and conditional exon 8-knockout mice that we will generate will give rise to interesting phenotype since mice with over-expression of *NF2* isoform lacking exon 2 or with the deletion of exon 2 in Schwann cells displayed Schwann cell hyperplasia and schwannoma formation (Giovannini et al., 1999, 2000).

### Aim 3: Examination of the Potential Role of Differential Polyadenylation of *NF2* Transcripts.

Task 9: Previously we have constructed expression plasmids for the three human *NF2* cDNAs of 6.1, 3.1, and 2.7 kb in length. These cDNAs carrying different polyadenylation signal sequences. We have also inserted a hemagglutinin (HA) epitope to the N-terminus of the *NF2*-coding region to facilitate protein detection. We have transfected these *NF2* cDNA expression plasmids into RT4 schwannoma, SK-N-AS neuroblastoma, and 293 kidney cells and are in the process of comparing the RNA half-life expressed from these *NF* cDNA expression constructs,

Task 10: Accomplished in previous year 2. A series of 3' unidirectional deletion derivatives from the 6.1-kb *NF2* cDNA expression plasmid has been generated for Task 11.

Task 11: The 6.1-, 3.1- and 2.7-kb *NF2* cDNA expression plasmids carrying different lengths of the 3' UT sequences were transfected into RT4 schwannoma, SK-N-AS neuroblastoma, and 293 kidney cells. We are conducting Western Blot analysis to detect the level of *NF2* protein expression from these constructs. These experiments should allow us to examine whether the 3' UT sequence of the *NF2* gene plays any role in protein translation.

Task 12: During the past year, we presented two research abstracts to two national meetings. We also submitted three papers; one was published (Chang et al., 2005), the 2<sup>nd</sup> one is in-press (Neff et al., 2005), and the 3<sup>rd</sup> one under-review (Akhmametyeva et al., 2005). Detailed description of our findings is presented in the following sections and in Appendices.

## KEY RESEARCH ACCOMPLISHMENTS:

(1) We have confirmed our findings that vestibular schwannomas express a distinct pattern of alternatively spliced *NF2* transcripts lacking specific exons. The findings were reported in the papers Chang et al. (2005) and Neff et al. (2005)

(2) A key accomplishment during this reporting period is the characterization of the *NF2* promoter expression pattern during embryonic development. By a combination of approaches, transgenic analysis, *in situ* hybridization, and antibody staining, we show that *NF2* is an early expression marker. *NF2* promoter expression was detected as E5.5 and strong promoter activity was seen in the embryonic ectoderm. *NF2* promoter continued to be actively expressed in the neural ectoderm and its derived neural tissues throughout mid-embryogenesis. Importantly, we observed robust *NF2* promoter activity in the developing brain and in sites containing migrating cells including the neural tube closure, branchial arches, dorsal aorta, and paraaortic splanchnopleura. Furthermore, we noted a transient change of *NF2* promoter activity during neural crest cell migration. While little *NF2* promoter expression was detected in premigratory neural crest cells at the dorsal ridge region of the neural fold, significant activity was seen in the neural crest cells already migrating away from the dorsal neural tube. In addition, we detected considerable *NF2* promoter activity in various *NF2*-affected tissues such as acoustic ganglion, trigeminal ganglion, spinal ganglia, optic chiasma, the ependymal cell-containing tela choroidea, and the pigmented epithelium of the retina. The *NF2* promoter expression pattern during embryogenesis suggests a specific regulation of the *NF2* gene during neural crest cell migration and further support the role of merlin in cell adhesion, motility, and proliferation during development. A manuscript describing these findings has been prepared for publication (Akhmametyeva et al., 2005).

(3) Another key effort was devoted to the generation of a conditional targeting allele of the mouse *Nf2* gene. By using the Cre:*loxP* approach, we constructed a conditional gene targeting vector containing the exon 8 sequence of the *Nf2* gene flanked by two *loxP* recombination sites. By homologous recombination in mouse embryonic stem cells, we have successfully identified several ES clones carrying the *Nf2*<sup>*lox8*</sup> allele. These ES clone will be used to create an exon 8 knockout mouse. This experiment should allow us to examine the functional importance of exon 8 which was alternatively spliced in vestibular schwannoma.

## REPORTABLE OUTCOMES:

Two research abstracts were presented to national meetings during the past year. Also, three research papers were submitted; one was published, the 2<sup>nd</sup> one has been accepted for publication, and the third one is under review.

### Abstracts

- (1) Akhmametyeva, E.M., H. Luo, M. Mihaylova, D.B. Welling, and L.-S. Chang. 2005. The Neurofibromatosis Type 2 Gene Is Strongly Expressed in Neural Ectoderm and Pigmented Epithelium of the Retina During Early Development. The CTF International Consortium for Molecular Biology of NF1 And NF2, Aspen, CO.

*The objective of this study is to define the expression pattern of the NF2 gene during embryonic development. Two approaches were undertaken. First, we generated four lines of transgenic mice*



carrying a 2.4-kb NF2 promoter-driven the lacZ gene with a nuclear localization signal. Whole-mount X-gal staining of embryos at various days post coitus (pc) was conducted and tissue sections were analyzed. Second, we performed in situ hybridization using various Nf2 cDNA fragments as probes. We showed that the 2.4-kb NF2 promoter could direct  $\beta$ -galactosidase ( $\beta$ -gal) expression in transgenic embryos at as early as 5.5 days pc.  $\beta$ -gal staining was observed in embryonic endoderm and ectoderm, but not in the extraembryonic tissues. At 6.5 days pc,  $\beta$ -gal was expressed at high levels in embryonic ectoderm particularly in mitotic cells.  $\beta$ -gal staining was also detected in some endoderm and at lower levels in mesoderm. At 7.5 days pc, strong  $\beta$ -gal expression continued to be seen in embryonic ectoderm, and staining was more saturated in the head and neural folds of the forming neural plate. At 8.5 days pc, the highest level of  $\beta$ -gal expression was found in neural tube with the maximal staining at the cranial end. In addition,  $\beta$ -gal staining was detected in neural crest, the developing heart, and blood vessels. As embryos matured, strong  $\beta$ -gal expression was seen in the pigment epithelium of the retina. Less-intense  $\beta$ -gal staining was also found in the ganglion, liver, mesonephric system, genital ridge, and skin. In situ hybridization of embryos at 7.5, 8.5, and 9.5 days pc showed that Nf2 expression was widely distributed in both the embryonic and extraembryonic tissues. The broad expression pattern of the NF2 gene during embryogenesis suggests that the merlin protein is important for multiple tissue development.

- (2) Akhmametyeva, E.M., H. Luo, M. Mihaylova, and L.-S. Chang. 2005. Developmental Expression of the Neurofibromatosis 2 Gene Promoter in Transgenic Mice. Pediatric Academic Societies' Annual Meeting, Washington, D.C.

*Our senior research associate Dr. Elena M. Akhmametyeva presented this research abstract to the Annual International Pediatric Research Meeting. The findings presented in this abstract were similar to those described in abstract #3 presented to the 2005 CTF International Consortium for the Molecular Biology of NF1, NF2, and Schwannomatosis*

#### Publication and Manuscript

- (1) Chang, L.-S., E.M. Akhmametyeva, M. Mihaylova, H. Luo, S. Tae, B. Neff, and D.B. Welling. 2005. Dissecting the Molecular Pathways in Vestibular Schwannoma Tumorigenesis. Recent Research Development in Genes & Genomes 1: 1-33.

*The NF2 tumor suppressor gene is frequently mutated in all three types of vestibular schwannomas. Analysis of NF2 function and regulation has provided the first step toward the better understanding of the molecular mechanism of schwannoma tumorigenesis. In this review, we summarized our findings on the regulation of expression of the NF2 gene at the transcriptional and post-transcriptional level. We also described our transgenic study to examine NF2 promoter expression during early embryonic development. The NF2 gene encodes a protein termed merlin/schwannomin, which shares a high degree of homology with the *ezrin*, *radixin*, and *moesin* (ERM) proteins. Genetic knockout experiments have shown that the NF2 gene product is a tumor suppressor for Schwann cell and meningeal cells. By using cDNA microarray analysis, we identified genes and pathways that were deregulated in vestibular schwannomas. Furthermore, we have revealed potential underlying molecular differences among the types of vestibular schwannomas. It is anticipated that further analysis of the information will lead to the development of novel pharmaco-therapeutics, offering alternatives to the current options of untreated observation of tumor growth, stereotactic radiation, or surgical removal.*

- (2) Neff, B.A., D.B. Welling, E.M. Akhmametyeva, and L.-S. Chang. 2005. The Molecular Biology of Vestibular Schwannomas: Dissecting the Pathogenic Process at the Molecular Level. Otology Neurotology, In Press.

*Recent advances in molecular biology have led to a better understanding of the etiology of vestibular schwannomas. Mutations in the neurofibromatosis type 2 tumor suppressor gene (NF2) have been identified in vestibular schwannomas. We reviewed the clinical characteristics of vestibular schwannomas and neurofibromatosis type 2 (NF2) syndromes and related to alterations*



in the NF2 gene. Additionally, we discussed potential functions of merlin, the protein product of the NF2 gene. The discovery of how merlin interacts with other proteins may lead to a better understanding of NF2 function. Understanding merlin's interactions with other proteins, signaling pathways, and regulation of the NF2 gene will lead to the development of novel treatments for vestibular schwannomas. By using recently developed cDNA microarray technology, genes or pathways that are deregulated in vestibular schwannomas have been identified. Ultimately, drug therapies will be designed to stop schwannoma progression. This will offer alternatives to the current options of untreated observation of tumor growth, stereotactic radiation, or surgical removal. Furthermore, it may also be possible to develop targeted therapy that may shrink or altogether eradicate preexisting tumors.

- (3) Akhmametyeva, E.M., M. Mihaylova, H. Luo, Sadeq Kharzai, D.B. Welling, and L.-S. Chang. 2005. Regulation of the *Neurofibromatosis 2* Gene Promoter Expression during Embryonic Development. Submitted to Dev. Dyn.

*To better understand NF2 expression in vivo, we generated transgenic mice carrying a 2.4-kb NF2 promoter driving  $\beta$ -galactosidase ( $\beta$ -gal) with a nuclear localization signal. Whole-mount embryo staining revealed that the NF2 promoter directed  $\beta$ -gal expression as early as embryonic day E5.5. Strong expression was detected at E6.5 in the embryonic ectoderm containing many mitotic cells.  $\beta$ -gal staining was also found in parts of embryonic endoderm and mesoderm. The  $\beta$ -gal staining pattern in the embryonic tissues was corroborated by in situ hybridization analysis of endogenous Nf2 RNA expression. Importantly, we observed strong NF2 promoter activity in the developing brain and in sites containing migrating cells including the neural tube closure, branchial arches, dorsal aorta, and paraaortic splanchnopleura. Furthermore, we noted a transient change of NF2 promoter activity during neural crest cell migration. While little  $\beta$ -gal activity was detected in premigratory neural crest cells at the dorsal ridge region of the neural fold, significant activity was seen in the neural crest cells already migrating away from the dorsal neural tube. In addition, we detected considerable NF2 promoter activity in various NF2-affected tissues such as acoustic ganglion, trigeminal ganglion, spinal ganglia, optic chiasma, the ependymal cell-containing tela choroidea, and the pigmented epithelium of the retina. The NF2 promoter expression pattern during embryogenesis suggests a specific regulation of the NF2 gene during neural crest cell migration and further support the role of merlin in cell adhesion, motility, and proliferation during development.*

## CONCLUSIONS:

Vestibular schwannomas express a distinct pattern of alternatively spliced NF2 transcripts lacking specific exons, suggesting that these alternatively spliced exons may be important for NF2 function. The results from the analysis of the NF2 promoter expression pattern during embryogenesis suggest a specific regulation of the NF2 gene during neural crest cell migration and further support the role of merlin in cell adhesion, motility, and proliferation during development. Transgenic and conditional knockout mice are being generated to address whether the alternative splicing NF2 isoforms preferentially expressed in schwannomas possess any additional properties conducive to tumor formation *in vivo*.

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- Akhmametyeva, E.M., M. Mihaylova, H. Luo, D.B. Welling, and L.-S. Chang. 2005. Regulation of the *Neurofibromatosis 2* (NF2) Promoter Expression during Early Embryonic Development. Submitted to Dev. Dyn.
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- Neff, B.A., D.B. Welling, E. Akhmametyeva, and L.-S. Chang. 2005. The molecular biology of vestibular schwannomas: Dissecting the pathogenic process at the molecular level. *Laryngoscope*, In Press.
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## ABSTRACT

Neurofibromatosis type 2 (NF2) is associated with a homozygous inactivation of the neurofibromatosis 2 gene (*NF2*). Despite intense study of the *NF2* gene, the mechanism by which the *NF2* tumor suppressor acts to prevent tumor formation is not well understood. The *NF2* transcripts undergo alternative splicing, generating a series of mRNA isoforms lacking one or more exons. Presently, the role of alternative splicing of *NF2* mRNAs is not understood. The *NF2* transcripts are also terminated at different polyadenylation sites. The role of this differential polyadenylation is not known. The goal of this research is to examine the role of alternative splicing and differentiation polyadenylation of the *NF2* gene. During this reporting period, we have confirmed our findings that vestibular schwannomas express a distinct pattern of alternatively spliced *NF2* transcripts lacking specific exons. We have carried out a detailed analysis of *NF2* expression during embryonic development and in the tissues affected by NF2 using the transgenic, *in situ* hybridization and antibody staining approaches. We show that *NF2* is an early expression marker. *NF2* promoter expression was detected as E5.5. Strong *NF2* promoter activity was seen in the embryonic ectoderm and in all NF2-affected tissues examined. Importantly, we observed strong *NF2* promoter activity in the developing brain and in sites containing migrating cells including the neural tube closure and branchial arches. Furthermore, we noted a transient change of *NF2* promoter activity during neural crest cell migration. While little *NF2* promoter expression was detected in premigratory neural crest cells at the dorsal ridge region of the neural fold, significant activity was seen in the neural crest cells already migrating away from the dorsal neural tube. The *NF2* promoter expression pattern during embryogenesis suggests a specific regulation of the *NF2* gene during neural crest cell migration and further support the role of merlin in cell adhesion, motility, and proliferation during development. By using the conditional gene targeting approach, we have also created an *Nf2<sup>fllox8</sup>* allele and are in the process of generating a conditional exon 8 knockout mouse. These transgenic and knockout experiments should allow us to study the function of schwannoma-expressed *NF2* isoforms *in vivo*.

## APPENDICES:

### Publications and Papers In Press

- (1) Chang, L.-S., E.M. Akhmametyeva, M. Mihaylova, H. Luo, S. Tae, B. Neff, and D.B. Welling. 2005. Dissecting the Molecular Pathways in Vestibular Schwannoma Tumorigenesis. *Recent Research Development in Genes & Genomes* 1: 1-33.
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### Submitted Manuscripts

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## Dissecting the molecular pathways in vestibular schwannoma tumorigenesis

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### Abstract

*Human vestibular schwannomas, which are benign Schwann cell tumors originating from the eighth nerve in the posterior cranial fossa, continue to cause morbidity associated with hearing loss, balance dysfunction, facial paralysis and paresthesias, and occasionally life-threatening brainstem compression. Vestibular schwannomas can be divided into three general categories including unilateral spontaneous vestibular schwannomas, neurofibromatosis type 2 (NF2)-associated vestibular schwannomas, and cystic type schwannomas. Recent advances in molecular oncology have led to the discovery of the neurofibro-*

*matosis type 2 gene (NF2), whose mutation has been found in all three types of vestibular schwannomas. Expression of the NF2 gene is regulated at the transcriptional and post-transcriptional level, and its expression is essential during early embryonic development. The NF2 gene encodes a protein termed merlin/schwannomin, which shares a high degree of homology with the ezrin, radixin, and moesin (ERM) proteins. Genetic knockout experiments have shown that the NF2 gene product is a tumor suppressor for Schwann cell and meningeal cells. Analysis of cDNA microarray has revealed potential underlying molecular differences among the types of vestibular schwannomas. Avenues for the development of potential future therapies have begun to emerge.*

## **Introduction**

Vestibular schwannomas are histologically benign tumors of the neural sheath that originate from the superior or inferior vestibular branches of cranial nerve VIII. The term “vestibular schwannoma” is preferred over the more commonly used term “acoustic neuroma” because these tumors are neither neuromas, nor do they arise from the acoustic (cochlear) nerve (170). The tumors can lead to profound hearing loss, balance dysfunction, facial nerve paralysis, brainstem compression, hydrocephalus, and if left untreated, death. In a silent, off-balance world with impaired facial expression, these patients are directly affected in their ability to communicate and interact with others. Currently there are no known medical treatments for these tumors. Sensitive diagnostic techniques such as magnetic resonance imaging (MRI) are diagnosing these tumors at early stages. Surgical excision through a craniotomy or stereotactic radiation are the current treatments of choice. This review aims to address the types of vestibular schwannomas and molecular mechanisms underlying schwannoma tumorigenesis. Potential sources of phenotypic variation among schwannoma tumor types have been investigated and potential deregulated signaling pathways identified will be discussed with respect to the future development of novel pharmaco-therapeutics.

## **Vestibular schwannoma types**

Three types of vestibular schwannomas, which have distinct clinical features, are encountered in clinical practice including unilateral sporadic vestibular schwannomas, bilateral or NF2-associated schwannomas, and cystic schwannomas (238). Vestibular schwannomas, typically slow growing and non-malignant, most commonly occur as sporadic unilateral solid tumors with an annual incidence of about 1 in 20,000 population. Patients with vestibular schwannomas usually present with unilateral tinnitus, hearing loss and imbalance. These tumors can lead to deafness, facial nerve paralysis, brainstem

compression, hydrocephalus, and death, if left untreated. The mean age at onset in our series is 49 years of age and there is a slight female predominance without ethnic predilection (238). NF2-associated vestibular schwannomas and cystic schwannomas are both distinctly less common, each occurring at approximately 4% of the rate of unilateral schwannomas (30-32). NF2-associated and cystic tumors are more likely to be multi-lobulated than unilateral sporadic tumors.

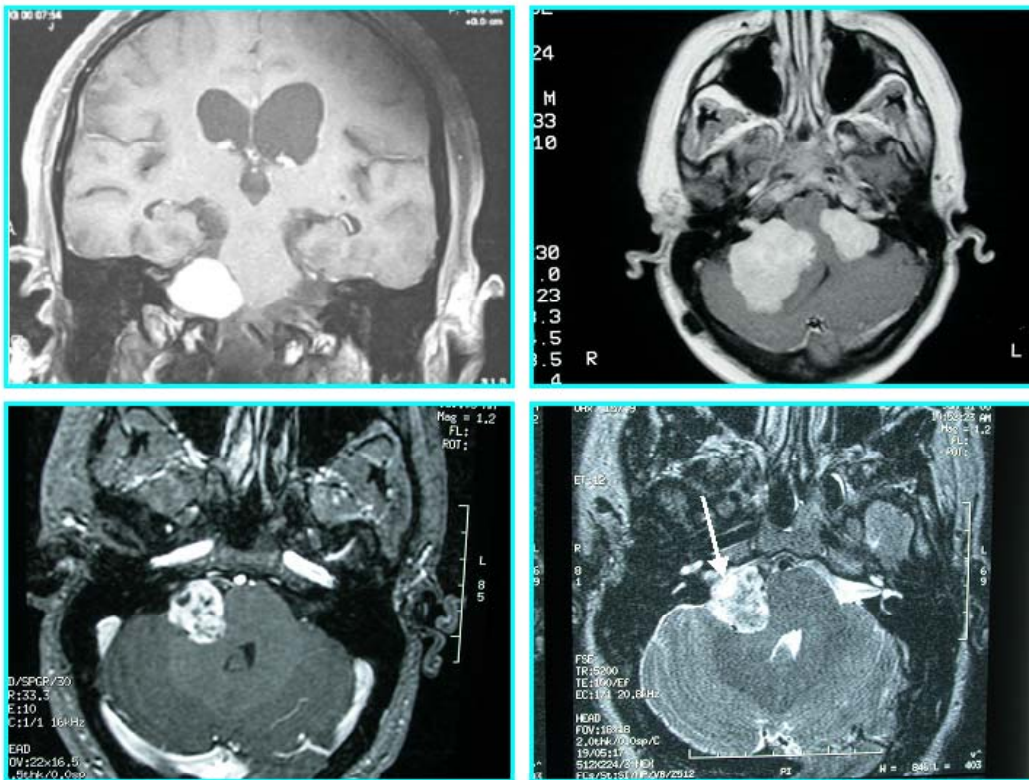
NF2 is a highly penetrant, autosomal dominant disorder (26). The hallmark of NF2 is the development of bilateral vestibular schwannomas. Other disease features include cranial meningiomas, ependymomas, spinal schwannomas and presenile lens opacities (61,109,111,148). NF2 is an extremely debilitating disease, leading to a decreased life expectancy in those afflicted. It has a prevalence estimated at approximately 1 in 40,000 (10,53). There is no known ethnic predilection. Affected individuals often show eighth-nerve dysfunction beginning in early adulthood including tinnitus, bilateral profound hearing loss, and ataxia. Bilateral facial nerve paralysis is an additional debilitating event. The mean age at presentation for NF2-associated vestibular schwannomas is 20 to 21 years of age (54,174). Occasionally the onset will be delayed into the 5th or 6th decade of life, but it may also present in early childhood (150). Patients who inherit an abnormal copy of the neurofibromatosis 2 gene (*NF2*) on chromosome 22q12 (245) have a 95% chance of developing bilateral vestibular schwannomas. However, about one half of the cases have no family history of NF2 and thus represent new germline mutations. NF2 is now recognized as a disease that is distinctly different from neurofibromatosis type 1 (NF1) or von Recklinghausen's disease. NF1, which is associated with multiple peripheral neuromas, is caused by a mutation in the neurofibromatosis 1 gene (NF1) located on the long arm of chromosome 17 (for recent reviews, see 9,40,142).

Cystic vestibular schwannomas are a particularly aggressive group of unilateral schwannomas, which invade the surrounding cranial nerves, splaying them throughout the tumor. Cystic vestibular schwannomas are associated with either intra-tumoral or extra-tumoral cysts, which develop in the loosely organized Antoni B tissues. In addition, a higher degree of nuclear atypia is seen in cystic tumors (33,110). Careful distinction must be drawn between truly cystic schwannomas and the more common heterogeneous schwannomas, which are not as aggressive in their clinical behavior. Cystic tumors may grow rapidly and are very difficult to manage, because of the high rate of hearing loss and facial nerve paralysis that occurs subsequent to their removal. When cystic tumors are removed surgically, only 35% of patients maintain a House-Brackmann grade I or II (normal or mild dysfunction) facial function postoperatively (30-32). These results are unfavorable when compared to surgical excision of NF2-associated vestibular schwannomas and sporadic



unilateral vestibular schwannomas which result in House-Brackmann grade I or II facial function in 66% and 90% of patients, respectively (238). In addition, surgical removal of cystic tumors resulted in a 41% rate of complete facial nerve paralysis (House-Brackmann grade VI) as compared to solid unilateral schwannomas of a similar size in which complete paralysis only occurred 27% of the time (62). The results from stereotactic radiation have been similarly disappointing for cystic tumors. These tumors are also much more likely to have continued growth and facial nerve paralysis with stereotactic radiation treatments than either unilateral spontaneous or NF2-associated schwannomas (177,200,214).

MRI distinguishes clearly among the three types of vestibular schwannomas (Figure 1). Cystic regions within cystic schwannomas are signal intense on T2-weighted images without contrast enhancement, while non-cystic components of the tumors enhance on T1 images with gadolinium, in a



**Figure 1.** MRI images of vestibular schwannomas. (A) Coronal T1-weighted image with gadolinium enhancement demonstrating a right-sided sporadic vestibular schwannoma. (B) Axial T1-weighted image with gadolinium enhancement illustrating bilateral NF2-associated vestibular schwannomas. (C) Axial T1-weighted image with gadolinium enhancement showing a right-sided vestibular schwannoma with areas of central low intensity corresponding to cysts within the tumor. (D) Axial T2-weighted image displaying a right-sided cystic vestibular schwannoma with focal areas of increased signal intensity that correspond to intra-tumoral cysts (arrow).

similar manner to those seen in unilateral and NF2-associated schwannomas. The irregular appearance of heterogeneous tumors on contrast enhanced MRI can be accounted for by hemosiderin deposits, which correlates with increasing tumor size (169).

Although quite distinct clinically and radiographically, the underlying molecular differences among the types of vestibular schwannomas are not understood. Furthermore, the optimal treatment regimens are not known because of a lack of understanding of fundamental tumor biology and a lack of rigorous clinical outcomes studies.

## ***NF2* gene, gene product, and mutation in vestibular schwannomas**

The gene associated with *NF2* was cloned by positional cloning and named the *NF2* gene, which encodes a protein named 'schwannomin' (a word derived from schwannoma, the most prevalent tumor seen in NF2) by Rouleau et al. (190) or 'merlin' (for moesin-ezrin-radixin like protein) by Trofatter et al. (229). To honor both groups, to retain the significance associated with each name, and to unify the nomenclature in the literature, we recommended that the *Nf2* gene product be called 'schwannomerlin' (240). However, for simplicity, we refer to the *NF2* gene product as merlin in this review.

Merlin shares a high degree of homology with the ezrin, radixin, and moesin (ERM) proteins (190,229). The ERM proteins are members of the erythrocyte protein 4.1-related superfamily, linking the actin cytoskeleton to the plasma membrane (2). The proteins belonging to this family all have similar designs with an N-terminal globular FERM domain (F for 4.1 protein) (35), followed by an  $\alpha$ -helical stretch and then by a charged C-terminus. The overall structure of merlin is similar to that of the ERM proteins. Biochemical and biological analysis indicates that the highly conserved FERM domain and the unique C-terminus of the merlin protein are important for its function. However, unlike the ERM proteins, which seem to facilitate growth, merlin exerts a growth suppression effect (78).

The isolation of the *NF2* gene facilitates the identification of mutations within the *NF2* gene, the relative frequency of mutations in each exon, and the specific clinical expression associated with specific *NF2* mutations in vestibular schwannomas and other NF2-associated tumors. *NF2* mutations have been identified in NF2-associated vestibular schwannomas, sporadic unilateral schwannomas, and cystic schwannomas (6,14,16-19,43-44,55-56, 75,77,82,96,98-103,112,119-122,135-136, 143-145, 155-156,158,165,176,190, 192,194,196-198,220-221,229-230,238,242,243,255). Additionally, mutations within the *NF2* gene have been frequently identified in meningiomas and

occasionally identified in other tumor types such as mesotheliomas (14,15,41,44,127,136,155,193,207).

Attempts have been made to correlate clinical expression with specific *NF2* mutations in vestibular schwannomas and other *NF2*-associated tumors. A number of somatic mutations and their specific clinical expression in sporadic unilateral tumors and *NF2* tumors have been characterized (14, 18, 19,44,55,75, 100-103, 119-122, 140, 143-144, 149, 155,175,192,196-198, 204, 221,230,242). In tumors from *NF2* patients, point mutations accounted for the majority of mutations, whereas small deletions accounted for the majority of mutations in the unilateral tumors. Initially, mutations, which cause truncation of the *NF2* protein, were reported to cause a more severe phenotype (99,103,175), while missense mutations or small in-frame insertions had been reported to associate with a mild phenotype (19,75,156,192,242). However, phenotypic variability within *NF2* families carrying the same germline mutation has also been reported (149). Although some missense mutations of the *NF2* gene have been associated with a milder phenotype, severe phenotypes have also been identified. Missense mutations within the  $\alpha$ -helical domain of merlin appear to be associated with a less severe phenotype than mutations within the conserved FERM domains (75). Likewise, large deletions may give rise to mild phenotypes as well (24). Given the heterogeneity of clinical response to various mutations, other yet unknown regulatory factors likely play a key role in the clinical manifestations in the various types of schwannomas.

It should be noted that not all vestibular schwannomas examined carry an identifiable *NF2* mutation. By performing an exhaustive alteration screening, including large deletions for the *NF2* gene, Zucman-Rossi et al. (255) reported the efficiency of mutation detection in *NF2* patients to be 84%. Thus, additional mechanisms for inactivation of the *NF2* gene in some *NF2* patients exist. The possibility of a modifier gene has been suggested (22-23). Also, the possibility of methylation or mutation in the *NF2* regulatory region has been postulated (29,116). In addition, the complexity of *NF2* transcripts generated by post-transcriptional alternative splicing and differential polyadenylation may be considered as possible means of inactivating the *NF2* gene (29).

Of clinical importance is the ability to completely identify a specific mutation in the *NF2* gene in a given family member because it allows the performance of direct DNA diagnostic testing on other family members, leading to an early, even presymptomatic diagnosis of affected patients. Early detection makes a distinct difference in the ability to successfully treat the patients (67). Decreased morbidity and mortality are associated with early intervention. Ultimately, facial nerve function and hearing preservation are directly correlated with smaller tumor size and early detection. *NF2* patients now often suffer profound bilateral deafness (241). Early detection however,

may allow hearing preservation by early surgical intervention. Additionally hearing restoration by cochlear implantation is only possible in NF2 patients diagnosed early enough to allow surgical sparing of the cochlear nerve. Cochlear implantation, if possible, is likely to achieve superior hearing results when compared to auditory brainstem implants (75,90). Individuals who have not inherited the abnormal *NF2* gene will be spared repeated medical evaluations and expensive imaging studies (125). All of these factors suggest that further refinement in the diagnosis and treatment of NF2 is necessary.

## **Merlin: A tumor suppressor in schwann cells**

Over-expression of the *NF2* gene in mouse NIH3T3 fibroblasts or rat RT-4 schwannoma cells can limit cell growth (75,78,141,210) and suppress transformation by a ras oncogene (228). The growth control of certain Schwann cells and meningeal cells is abrogated by the loss of *NF2* function, suggesting that *NF2* deficiency disrupts some aspect of intracellular signaling that leads to a signal for cell proliferation. Like the ERM proteins, merlin is expressed in a variety of cell types where it localizes to areas of membrane remodeling (21), particularly membrane ruffles (70). Schwannoma cells from NF2 tumors have dramatic alterations in the actin cytoskeleton and display abnormalities in cell spreading (178). These results suggest that merlin may play an important role in regulating both actin cytoskeleton-mediated processes and cell proliferation.

A few merlin-interacting proteins have been identified. These include the ERM proteins, F-actin, microtubules,  $\beta$ II-spectrin, hyalurin receptor CD44,  $\beta$ 1-integrin, paxillin,  $\beta$ -fodrin, syntenin, the regulatory cofactor of  $\text{Na}^+$ - $\text{H}^+$  exchanger (NHE-RF), SCHIP-1, hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), p21-activated kinase 1 and 2 (Pak1 and Pak2), Rac1, and RI $\beta$  subunit of protein kinase A (1,58,70,72,74,86,94,106,117-118,162,164,167,173,195,205-206,209-211,227,249-250). Presently, how these protein-protein interactions relate to the tumor suppressor activity of merlin is largely not understood. The association of merlin with the CD44 hyaluronic acid receptor and  $\beta$ 1-integrin raises the possibility that merlin might function as a molecular switch through signals from the extracellular matrix (86,162,212). Intriguingly, we have found that several extracellular matrix proteins are differentially regulated in vestibular schwannomas (243).

Mice carrying a deletion of the *Nf2* allele have been produced. Heterozygous *Nf2*-knockout mice develop a variety of malignant metastatic tumors with osteosarcomas at a high frequency, and fibrosarcoma and hepatocellular carcinoma at an increased, but lower frequency (152). Almost all of the tumors exhibit loss of the wild-type *Nf2* allele, indicating that merlin has a classical tumor suppressor function. However, none of the heterozygous

*Nf2* mice develop vestibular schwannomas or other non-tumoral manifestations of human NF2. Homozygous *Nf2* mutant mouse embryos fail in development at day 6.5~7 of gestation and die immediately prior to gastrulation, displaying poorly organized extra-embryonic ectoderm (153). These results suggest that merlin is essential during embryonic development. By using Cre-mediated excision of exon 2 in Schwann cells targeted by a myelin basic protein P0 promoter, conditional *Nf2*-knockout mice have been produced and display characteristics of NF2, including schwannomas, Schwann cell hyperplasia, cataracts, and osseous metaplasia (66). Although these results argue that loss of merlin is sufficient for schwannoma formation *in vivo*, none of the lesions observed in these mice involve the vestibular nerve. This is in contrast to those vestibular schwannomas commonly found in patients with NF2. In addition, meningiomas, a frequent manifestation of the human NF2 disease, were not observed in the conditional homozygous *Nf2* knockout mice suggesting that meningioma progenitor cells may not be permissive to the P0 promoter. The use of the *NF2* gene promoter to direct proper temporal and spatial expression of the Cre recombinase to Schwann cells and other cell lineages to generate additional conditional knockout mice may allow validation of this hypothesis.

## **Transcriptional and post-transcriptional regulation of the NF2 gene**

The human *NF2* gene spans over 90-kb DNA on chromosome 22 (239,255). To better understand *NF2* gene expression and regulation, we previously mapped the 5' and 3' ends of the *NF2* transcript (29). The human *NF2* transcripts initiate at multiple positions, with the major transcription initiation site mapped at position -423, relative to the A residue of the ATG translation start codon designated as +1. The proximal 5' flanking region of the human *NF2* gene is G+C rich and no consensus TATA sequence is present in the region 30 bp upstream of the major transcription initiation site. However, several potential binding sites for transcription factors AP2, CTF, E2F, EKLF, GCF, HIP1, LEF/TCF-1, NF-S, peroxisome proliferator-activating receptor (PPAR), *c-Sis*/PDGF, and Sp1 are present in the 5' flanking region (29).

The NF2 protein is expressed in a variety of cell types (29,79,97,153,190,229). Consistently, our promoter analysis shows that a 2.4-kb 5' flanking DNA of the human *NF2* gene directs the efficient expression of a reporter enzyme in several human cell types (29,239). Deletion analysis reveals a core promoter region extending 400 bp from the major transcription initiation site. Multiple positive and negative elements within the 5' flanking region appear to be important for *NF2* promoter expression. In particular, a G/C-rich sequence located at position -58 to -46, which can be bound by the Sp1 transcription factor, serves as a positive regulatory element. Co-

transfection experiments in *Drosophila* SL2 cells demonstrate that Sp1 can activate the *NF2* promoter through the G/C-rich sequence (29).

To examine if the *NF2* promoter is sufficient to direct expression to Schwann cells and other cell types, we have produced transgenic mice carrying a 2.4-kb *NF2* promoter-driven  $\beta$ -galactosidase ( $\beta$ -gal) construct. Whole mount *X-gal* staining of transgenic embryos detected  $\beta$ -gal expression at early embryonic development (Akhmametyeva, E., M. Mihaylova, and L.-S. Chang, unpublished data). At 10.5 days post coitus (p.c.), strong  $\beta$ -gal staining was detected in the developing neural tissues, including those developing into the brain and the cells surrounding the spinal nerves. A broad  $\beta$ -gal staining pattern was seen in transgenic embryos at 12.5 days p.c. and older. This early embryonic expression pattern of the *NF2* promoter is consistent with the phenotype of embryonic lethality seen in *Nf2*-knockout mice (153). Together, these results suggest that *NF2* may have a broad role during early development.

We have also found that the promoter region of the human *Nf2* gene could function bi-directionally (Chang, L.-S. and E. Akhmametyeva, unpublished data). When a *NF2* promoter-luciferase construct containing the 600-bp *NF2* promoter DNA placed in the antisense orientation with respect to the luciferase expression cassette, was used in transfection, it gave rise to very strong promoter activity, which was higher than that of the 600-bp *NF2* promoter in the sense configuration. Further analysis on the bi-directional transcription architecture of the *NF2* locus should allow us to better understand the regulatory mechanism interplayed between two closely spaced promoters and possibly transcription units.

The *NF2* coding region encompasses 17 exons (190,229,239,255). Northern blot analysis detects multiple *NF2* RNA species of 6.1, 3.9, and 2.7 kb in various human tissues and cell lines (29). We have shown that the complexity of *NF2* transcripts is attributed to multiple levels of regulation including multiple transcription initiation sites, alternative splicing, and differential usage of multiple poly(A) signal sequences (29). Various lengths of *NF2* cDNAs have also been isolated. The longest human *NF2* cDNA, containing all 17 exons, consists of 6,067 nucleotides (nt), which is consistent with the size of the major RNA species hybridized to the *NF2* probe. The cDNA has a 425-nt 5' untranslated (UT) region upstream from the ATG start codon, and a long 3' UT region of 3,869 nt.

*NF2* transcripts undergo alternative splicing in the coding exons (7,14,29,88,123,171,182,202) and possibly in the 3' UT region (Wu, Y., E. Akhmametyeva, and L.-S. Chang, unpublished data). So far, more than 10 alternatively spliced cDNA isoforms have been identified in various human cells and tissues. The most common isoforms expressed in these cells are the so-called "isoform II", containing all 17 exons, and "isoform I", which is

missing exon 16. Isoform I is a 595 amino-acid protein. Isoform II differs from isoform I only at the C-terminus. Due to the inclusion of exon 16 in the mRNA providing a new stop codon, isoform II encodes a 590 amino-acid protein that is identical to isoform I over the first 579 residues. Other alternatively splice *NF2* isoforms include those missing exon 2, 3, 8, 10, or 15, or multiple exons and those with insertion of sequences from intron 2 or 16 (7,14,29,88,123,171,182,202; Akhmametyeva, E. and L.-S. Chang, unpublished data).

We have also examined the expression of alternatively spliced *NF2* mRNA isoforms in *NF2*-associated schwannomas, sporadic unilateral schwannomas, and cystic schwannomas. While the expression pattern and relative frequency of alternatively spliced *NF2* transcripts were similar among the three types of schwannoma; however, some differences were found when the *NF2* transcripts from schwannomas were compared to those isolated in other human cell types. For example, in addition to isoforms I and II, the schwannomas expressed a high percentage of the *NF2* mRNA isoform lacking exons 15 and 16 (Akhmametyeva, E., D.B. Welling, and L.-S. Chang, unpublished data).

Presently, the role of alternative splicing of *NF2* mRNA is not well understood. It is important to note that these alternatively spliced *NF2* isoforms could potentially encode different sizes of merlin protein. Intriguingly, among all isoforms tested, only isoform I possesses growth inhibitory activity when assayed *in vitro* (Yao, S., E. Akhmametyeva, and L.-S. Chang, unpublished data). However, transgenic mice expressing the *NF2* isoform lacking exon 2 showed high prevalence of Schwann cell-derived tumors and Schwann cell hyperplasia (65). Also, it is not known why schwannomas preferentially express certain *NF2* isoforms. It is possible that the functional contribution of the *NF2* tumor suppressor may require a balanced expression of various isoform proteins in Schwann cells and/or other cell lineages (29,65). Alternatively, it is tempting to speculate that alternative splicing may be another mechanism for Schwann cells to inactivate merlin function and/or to generate isoforms that have additional properties conducive to tumor formation. We are presently conducting experiments to test these possibilities.

In addition to the longest full-length 6.1-kb *NF2* cDNA, we have also isolated two shorter *NF2* cDNAs that were terminated by different polyadenylation signal sequences within intron 16 (29). These results indicate that differential usage of multiple polyadenylation sites also contributes to the complexity of human *NF2* transcripts. Presently, the functional significance for the differential polyadenylation of *NF2* transcripts is not understood. The structural difference among these *NF2* transcripts argues that they may play some role in the regulation of *NF2* expression. Nevertheless, in conjunction



with tissue-specific alternative splicing (29,202), differential polyadenylation may further provide functional diversity of the *NF2* protein.

## **cDNA microarray analysis of vestibular schwannomas**

Carcinogenesis is a multi-stage phenomenon including both genetic and epigenetic alterations (for recent reviews, see 81,113,219,237). In addition to mutations in oncogenes and tumor suppressor genes, deregulated expression of genes involved in specific signaling pathways, damage surveillance, DNA repair or mitotic apparatus frequently occurs in human cancer. In recent years, large-scale gene expression analysis using cDNA microarray has been successfully utilized in evaluating a number of solid tumors and leukemias (4,5,20,36,39,45,46,48,51,57,59,68,76,80,85,87,89,92,93,95,105,107,114,128,129,133,134,137,138,146,154,161,165,166,172,179,186,199,201,208,215,216,222,226,243,244,246). Analysis of gene expression profiles using a variety of statistical algorithms to arrange tissues according to similarity in pattern of gene expression (64) has revealed differences among tumors, which are not distinguishable histologically. Molecular classification, rather than histological classification, has been shown to predict the response of specific tumor types to specific therapies (12,25,147,168,189,236,248). This genomic scale approach has helped to identify sub-classes of colon carcinoma, breast carcinoma, melanoma, leukemia and lymphomas (3,8,37,49,63,84,139,157,163,191,203,217,218,225,251). In several instances, microarray analysis has already identified genes that appear to be useful for predicting clinical behavior (11,13,27,34,52,69,71,91,108,115,180,185,188,213,232-235,247).

Vestibular schwannoma characteristics cannot be explained by the current understanding of gene mutation types alone. Investigating inter-tumor variability of gene expression profiles shows promise to help unravel the clinical differences among subtypes of vestibular schwannomas. Three complementary approaches can be used to evaluate the gene expression profile of vestibular schwannomas. The first is to identify pathways of tumorigenesis common to schwannomas regardless of clinical variation in tumor manifestations. The second is to find pathways and/or genes, which are differentially expressed in tumors of the same type, and the third is to compare normal tissue expression profiles with tumor tissues' profiles.

To better understand the pathways leading to schwannoma formation, we have utilized cDNA microarray analysis to evaluate gene expression profiles of vestibular schwannomas (129,243). Three sporadic vestibular schwannomas, two *NF2*-associated schwannomas, and three cystic schwannomas were compared to a normal vestibular nerve from a patient with a sporadic schwannoma. Of 25,920 genes or expressed sequence tags screened, 42 genes were significantly up-regulated by a factor of three or more in 6 of the 8

tumors examined. Additionally, multiple genes were found to be significantly down-regulated in the majority of vestibular schwannomas examined. Of these genes, eight genes involved with cell signaling and division were down-regulated. These include an apoptosis-related, putative tumor suppressor gene LUCA-15, which was down-regulated in seven of eight schwannomas, studied (243). The LUCA-15 gene maps to the lung cancer tumour suppressor locus 3p21.3 and encodes an RNA binding nuclear protein (47,50). A number of alternative LUCA-15 RNA splice variants have been isolated and display different abilities to either enhance or suppress apoptosis (159,160,185,223, 224). The LUCA-15 protein contains zinc fingers and possesses transcription-activation function (Luo, H. and L.-S. Chang, unpublished data).

Among the up-regulated genes, two mediators of angiogenesis, endoglin and osteonectin, were highly elevated in most, if not all, vestibular schwannomas examined (243). Endoglin, also called CD105, is a cell membrane glycoprotein, which is frequently over-expressed on tumor-associated vascular endothelium (60). Endoglin functions as an accessory component of the transforming growth factor- $\beta$  receptor complex and has been suggested to have prognostic significance in selected neoplasia. In addition, the potential usefulness of endoglin in tumor imaging and anti-angiogenic therapy has been well documented (60). Endoglin was found to be significantly up-regulated in all of the solid vestibular schwannomas but not in any of the cystic schwannomas examined (243). The difference in endoglin gene expression may be a key to unlocking why some schwannomas develop into the aggressive cystic phenotype. Osteonectin is a secreted glycoprotein that interacts with extracellular matrix proteins to decrease adhesion of cells from the matrix, thereby inducing a biological state conducive to cell migration. Osteonectin was elevated in all of the tumors studied and may be a target for potential therapies including angiogenesis inhibitors (243).

An example of a deregulated signaling pathway suggested by the microarray data is the retinoblastoma protein (pRb)-cyclin dependent kinase (CDK) pathway, which regulates G<sub>1</sub>-S progression (129). All schwannomas examined displayed deregulated expression of at least one of the genes involved in this pRb-CDK pathway. In addition, CDK2 was found to be down-regulated in 7 of 8 tumors.

We also found up-regulation of transforming factor RhoB all of the schwannomas examined (243). The Rho GTPase proteins share about 30% homology with Ras and are crucial regulators of the actin cytoskeleton, cell adhesion, and motility (reviewed in 28,38,151,184). Rho-family members play a critical role in Ras transformation (100,181,183,254), making them potential anticancer therapeutic targets. Unlike most members of the Rho family, RhoB is a growth factor- and oncogenic-induced immediate early gene product that is regulated during the cell cycle (104,252). RhoB, by itself, is weakly

oncogenic, but is required for the initiation and maintenance of transformation by the oncogenic Ras protein (131,181). In addition, RhoB is a critical target of farnesyltransferase inhibitors, a novel class of anti-tumor chemotherapeutics (124,131-132,253). The fact that RhoB expression is elevated in all schwannomas examined suggests that RhoB may be a downstream target of merlin in Schwann cells (243).

One important consideration in our cDNA microarray analysis is the use of the paired normal vestibular nerve as the control (129,243). Experiments using cultured Schwann cells may yield data for direct comparison with schwannoma tissue. However, the drawback of using a cell culture is that culture conditions could alter gene expression patterns. Also, limited division has been commonly observed with cultured Schwann cells and vestibular schwannoma cells. Nevertheless, a direct comparison of protein expression for CDK2, RhoB, osteonectin, and merlin in tissue sections together with quantitative real-time PCR analysis for CDK2 and LUCA-15 yielded results consistent with our cDNA microarray data when schwannoma tumors were compared with the normal vestibular nerve (129,243). For confirmation, we are presently conducting additional cDNA microarray analysis comparing multiple pairs of vestibular nerves, vestibular schwannomas, and cultured Schwann cells.

Microarray analysis of vestibular schwannomas has opened many new potential avenues of investigation. A more complete understanding of why *NF2* mutations lead to tumor formation is dependent upon understanding other interacting genes whose expression are deregulated during tumorigenesis. Investigating inter-tumor variability and comparing normal Schwann cells to schwannoma cells via gene expression profiles shows promise to help unravel the clinical differences among the types of vestibular schwannomas.

## Summary

Recent advances in molecular oncology have identified the *NF2* tumor suppressor gene, which is frequently mutated in all three types of vestibular schwannomas. Analysis of *NF2* function and regulation has provided the first step toward the better understanding of the molecular mechanism of schwannoma tumorigenesis. Functional genomics and proteonomics studies have generated vast amounts of information for translational approaches. It is anticipated that further analysis of the information will lead to the development of novel pharmaco-therapeutics, offering alternatives to the current options of untreated observation of tumor growth, stereotactic radiation, or surgical removal.

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**The Molecular Biology of Vestibular Schwannomas:  
Dissecting the Pathogenic Process at the Molecular Level**

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## ABSTRACT

Recent advances in molecular biology have led to a better understanding of the etiology of vestibular schwannomas. Mutations in the neurofibromatosis type 2 tumor suppressor gene (*NF2*) have been identified in vestibular schwannomas. The clinical characteristics of vestibular schwannomas and neurofibromatosis type 2 (*NF2*) syndromes will be reviewed and related to alterations in the *NF2* gene. Additionally, merlin, the protein product of the *NF2* gene, will be discussed. The discovery of how merlin interacts with other proteins may lead to a better understanding of *NF2* function. By using recently developed cDNA microarray technology, genes or pathways that are deregulated in vestibular schwannomas have been identified. Avenues for the development of potential future therapies will be highlighted.

## Introduction

Vestibular schwannomas are histologically benign tumors of the neural sheath that originate on the superior or inferior vestibular branches of cranial nerve VIII. The term “vestibular schwannoma” is preferred over the more commonly used term “acoustic neuroma” because these tumors are neither neuromas, nor do they arise from the acoustic (cochlear) nerve. They occur either as sporadic unilateral tumors or bilateral tumors; the development of bilateral vestibular schwannomas is the hallmark of neurofibromatosis type 2 (*NF2*). Various types of vestibular schwannomas can be loosely grouped into unilateral sporadic vestibular schwannomas, bilateral or *NF2*-associated schwannomas, and cystic schwannomas.

Unilateral schwannomas are the most common presentation, and they constitute 95% of all vestibular schwannomas. Sporadic vestibular schwannomas occur in approximately 10 persons per million per year (1,2). However, the true incidence may be higher, as highlighted by Anderson et al., who demonstrated an incidence of 7 unsuspected schwannomas per 10,000 brain MRI studies (0.07%) (3). Sporadic tumors usually occur in the 4<sup>th</sup> and 5<sup>th</sup> decades with a mean presentation of 50 years of age. Although histologically benign, schwannomas can, if large enough, cause hydrocephalus, brain stem compression, herniation, and death. Most commonly, however, they are associated with hearing loss, tinnitus, imbalance, and other symptoms related to compression of adjacent cranial nerves.

*NF2* is clinically an autosomal dominant disease that is highly penetrant (4). *NF2*-associated tumors account for about 5% of all vestibular schwannomas. Patients who inherit an abnormal copy of the *NF2* tumor suppressor gene have a 95% chance of developing bilateral vestibular schwannomas. However, about one half of the cases have no family history of *NF2*, and thus, they represent new germline mutations that were not inherited. Other disease features of *NF2* include intracranial meningiomas, ependymomas, spinal schwannomas, and presenile lens opacities (5-8). *NF2* is now recognized as a disease that is distinctly different from neurofibromatosis type 1 (*NF1*) or von Recklinghausen's disease. *NF1*, which is associated with multiple peripheral neuromas, is caused by a mutation in the *NF1* tumor suppressor gene on chromosome 17.

*NF2* is currently subdivided into three groups (9,10). The *Wishart* type has a more severe clinical presentation. In addition to bilateral vestibular schwannomas, patients often suffer from associated spinal tumors with a typical onset in the late teens or early twenties (11). The *Gardner* type has a later onset and a less severe presentation. Although patients present with bilateral schwannomas, the incidence of associated intracranial tumors is less common. (12). A more recently recognized third category of *NF2* has been termed *segmental NF2*. The cause of *segmental NF2* may be due to somatic mosaicism where a mutation occurs in embryogenesis rather than in the germline DNA; therefore, only a portion of the patients' cells carries the mutation (13,14). This is different from those patients with traditional *NF2* who inherit the mutation from their parent. Kluwe et al. recently estimated that mosaicism may account for 24.8% (58/233) of the *NF2* cases of any subtype among patients whose

parents did not display the disease (14). Patients with somatic mosaicism can display bilateral vestibular schwannomas if the postzygotic mutation occurred early in embryogenesis. However, they may also display an atypical presentation, or *segmental* NF2, in which the patient has a unilateral vestibular schwannoma and an ipsilateral, additional intracranial tumor, such as a meningioma, if the postzygotic mutation occurred late in development (13). Unlike the traditional forms of NF2, the risk of passing NF2 caused by mosaicism to future offspring is very low (15).

Schwannomatosis, a recently defined form of neurofibromatosis, is characterized by multiple schwannomas without any vestibular schwannomas, diagnostic of NF2. Patients with schwannomatosis frequently present with intractable pain rather than cranial nerve deficits. They do not develop other intracranial tumors or malignancies. MacCollin et al. noted that about one third of patients with schwannomatosis had tumors in an anatomically limited distribution, such as a single limb, several contiguous segments of spine, or one half of the body (16). Sporadic cases of schwannomatosis are as common as NF2, but few cases of familial schwannomatosis have been identified. This is in contradistinction to NF1 and NF2, which are autosomal dominant, highly penetrant syndromes that are frequently found clustered in families. The underlying molecular disruption in schwannomatosis is a pattern of somatic *NF2 gene* inactivation incompatible with NF1 or NF2, but this has not been completely defined.

Cystic vestibular schwannomas are a particularly aggressive group of unilateral, sporadic schwannomas, which invade the surrounding cranial nerves, splaying them throughout the tumors. Cystic vestibular schwannomas are associated with either intratumoral or extratumoral cysts which develop in the loosely organized Antoni B tissues. In addition, a higher degree of nuclear atypia is seen in cystic tumors (17,18). Careful distinction must be drawn between the truly cystic schwannomas and the very common heterogeneous schwannomas which are not as aggressive in their clinical behavior. Magnetic resonance imaging (MRI) distinguishes clearly between the solid and cystic vestibular schwannomas. Cystic regions of the tumors are hyper-intense on T2-weighted images, and the cysts do not enhance with gadolinium administration. The non-cystic component of the cystic tumors enhances with gadolinium in a manner similar to the unilateral and NF2-associated schwannomas (**figure I**). Cystic tumors may grow rapidly, and they are very difficult to manage due to the high rate of hearing loss and facial nerve paralysis that occurs after surgical removal (19). When compared to solid tumors of a similar size, the rate of complete facial nerve paralysis (House-Brachmann grade VI) with surgical removal of cystic tumors was 41%, as compared to 27 % for that of solid unilateral schwannomas (20). Cystic tumors are also more likely to have continued growth and facial nerve paralysis even with stereotactic radiation treatments than either the unilateral spontaneous or NF2- associated schwannomas (21,22).

Although the effectiveness of treatment with current surgical and radiation treatments for vestibular schwannomas are generally good, treatment-related morbidity continues to be problematic. The field of molecular biology is proposed as the discipline to advance the level of diagnosis and to improve the treatment of vestibular schwannomas. When applied to various neurotologic pathologies, “molecular neurotology” may soon develop as a medical discipline in a manner similar to the advent of surgical neurotology in the 1960’s. A brief review of the recent discoveries and advances in the molecular biology of vestibular schwannomas will follow.

## **The *NF2* gene**

The *NF2* gene was localized to chromosome 22 through a genetic linkage analysis (23). Subsequently, twenty-three patients from a large NF2 kindred were studied, and the *NF2* locus was further mapped close to the center of the long arm of chromosome 22 (22q12) (24). Following genetic and physical mapping, positional cloning studies led to the discovery of the *NF2* gene. In 1993, Trofatter et al. and Rouleau et al., independently identified the *NF2* gene, which is frequently mutated in NF2-related vestibular schwannomas (25,26). Since that time, mutations in the *NF2* gene have been

found not only in NF2 tumors but also in sporadic unilateral schwannomas and cystic schwannomas (**table I**) (27-34). Additionally, mutations within the *NF2* gene have been frequently identified in meningiomas and occasionally identified in other tumor types such as mesotheliomas (32-40).

## **NF2 mutations and their clinical correlation**

Several groups have attempted to correlate clinical expression of tumors with specific *NF2* mutations in vestibular schwannomas and other NF2-associated tumors. A number of somatic mutations and their specific clinical behavior in vestibular schwannomas have been characterized in sporadic unilateral tumors and NF2 tumors (27,29,30,32,33,35,41-57). We previously studied a series of patients who had vestibular schwannomas and found that the frequency, type, and distribution of *NF2* mutations were shown to be different between the sporadic and familial NF2 tumors (27). Mutations were identified in 66% of the sporadic cases, but in only 33% of the NF2 cases; therefore, the rate of detection of a mutation in unilateral schwannomas were significantly higher than that in familial schwannomas. Point mutations accounted for the majority of mutations identified in NF2 patients, whereas small deletions accounted for the majority of mutations found in the sporadic unilateral tumors (44,45,54).

Studies were also done to determine if the genotype could be a predictor of disease severity. The clinical phenotypes of NF2, Wishart and Gardner, were further examined, as well as a potential third phenotype, mosaic or segmental NF2. Deletion mutations that cause truncation of the *NF2* protein have been reported to cause a more severe phenotype in NF2 pedigrees (44,45,54), while missense mutations or small in-frame insertions in the *NF2*-coding region have been reported to associate with a mild phenotype (27,32,33,52,55,56). However, this has not held true in other studies, which showed that some missense mutations associated with a severe phenotype. In addition, missense mutations within the  $\alpha$ -helical domain of the *NF2* protein appear to associate with a less severe phenotype than those within the conserved ERM domain (58). This lack of genotype-phenotype correlation was also seen for large deletions which could give rise to mild phenotypes as well as the previously reported severe disease expression (59). Given the heterogeneity of clinical response to various types of mutations, no clear genotype to phenotype correlation has been established, and this is further evidenced by the fact that phenotypic variability within the NF2 families with the same mutation has been seen (60). By extensive screening of the *NF2* gene, Zucman-Rocci reported an 88% mutation detection rate in vestibular schwannomas; thus, additional mechanisms for inactivation of the *NF2* gene in some patients may exist (61). The possibility of a modifier gene has been suggested (62). Also, mutation or methylation in the regulatory region of the *NF2* gene has been suggested as a possible mechanism of gene inactivation (63,64). The complexity of *NF2* transcripts generated by post-transcriptional alternative splicing and differential polyadenylation may also be considered as possible means of inactivating the *NF2* gene (64).

## **The NF2 protein- structure and function**

The *NF2*-coding region encompasses 17 exons spanning 90 kilo base-pairs of DNA on chromosome 22 (25,26,61). It encodes a 595-amino acid protein product which has been named *merlin* (for moesin-ezrin-radixin like protein) or *schwannomin* (derived from schwannoma) (25,26). For simplicity, the *NF2* protein will be referred to as “merlin” in this manuscript.

Merlin shares a high degree of homology to the erythrocyte protein 4.1-related superfamily of proteins, which act to link the actin cytoskeleton to the plasma membrane. In particular, three proteins, *ezrin*, *radixin*, and *moesin*, referred to as the ERM family, share a great deal of structural similarity with merlin (26,65). The proteins belonging to this family all have a similar N-terminal globular domain, also known as the FERM domain, followed by an  $\alpha$ -helical stretch, and finally a charged carboxyl-terminus (66). The key functional domains of merlin may lie within the highly conserved FERM

domains and the unique C-terminus of the protein. The ERM proteins have been shown to be involved in cellular remodeling involving the actin cytoskeleton (67). These proteins bind actin filaments in the cytoskeleton via a conserved C-terminal domain and possibly via a second actin-binding site in the N-terminal half of the protein (68,69).

Like the ERM proteins, merlin is expressed in a variety of cell types where it localizes to the areas of membrane remodeling, particularly membrane ruffles, although its precise distribution may differ from the ERM proteins expressed in the same cell (70). Interestingly, schwannoma cells from NF2 tumors show dramatic alterations in the actin cytoskeleton and display abnormalities in cell spreading (71). These results suggest that merlin may play an important role in regulating both the actin cytoskeleton-mediated processes and cell proliferation (72). However, it should be noted that merlin has a growth suppression role, while other ERM-family members seem to facilitate cell growth.

### **Merlin acts as a tumor suppressor**

Over-expression of the *nf2* gene in mouse fibroblasts or rat schwannoma cells can limit cell growth (56,73,74) and suppress cell transformation by the ras oncogene (75). The growth control of certain Schwann cells and meningeal cells is lost in the absence of NF2 function, suggesting that NF2 mutations and merlin deficiency disrupt some aspect of intracellular signaling that leads to cellular transformation. These findings demonstrate merlin's ability to act as a tumor suppressor.

Furthermore, scientists have developed *nf2* knockout mice which were designed to be missing one or both copies of the *nf2* gene in the germline. Intriguingly, heterozygous *nf2* knockout mice go on to develop osteosarcomas, and less often, fibrosarcomas or hepatocellular carcinomas (76). Genetic analysis of these tumors shows that nearly all of them are missing both *nf2* alleles due to a mutation causing a loss of the second *nf2* allele. The fact that tumor growth occurs in the absence of both *nf2* alleles indicates that the *nf2* gene possesses a classical tumor suppressor function. However, none of the heterozygous *nf2* mice develop tumors or clinical manifestations associated with human NF2.

Homozygous *nf2* mutant mice, which are designed to be missing both *nf2* alleles also do not demonstrate clinical characteristics of human NF2, and the mutant embryos die at approximately seven days of gestation, indicating that a homozygous *nf2* mutation is embryonic lethal (77). Together with our preliminary data showing that the *nf2* gene is expressed early in embryogenesis (our unpublished data), these results indicate that the *nf2* gene product plays an important role during early embryonic development.

By engineering mice whose Schwann cells have exon 2 excised and removed from both *nf2* alleles, conditional homozygous *nf2* knockout mice have been produced which display some characteristics of NF2 including schwannomas, schwann cell hyperplasia, cataracts, and osseous metaplasia (78). Although these results argue that loss of merlin is sufficient for schwannoma formation *in vivo*, none of the tumors observed in these conditional knockout mice were found on the vestibular nerve. This is in contrast to those vestibular schwannomas commonly found in patients with NF2. Further work is needed to develop a mouse model that phenotypically displays schwannomas originating from the eighth cranial nerve.

### **Merlin cell signaling and regulation**

In addition to the actin cytoskeleton, merlin has been shown to associate with cell membrane domains, which are highly enriched in signaling molecules that regulate cellular responses to proliferative and antiproliferative stimuli (79). To date, several proteins that are likely to interact with merlin have been identified. These include the ERM proteins, hyaluronin receptor CD44, F-actin, paxillin, microtubules,  $\beta$ II-spectrin,  $\beta$ 1-integrin,  $\beta$ -fodrin, the regulatory cofactor of  $\text{Na}^+$ - $\text{H}^+$  exchanger (NHE-RF), SCHIP-1, hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), p21-activated

kinase 1 and 2 (Pak1 and Pak2), Rac1, and RIB subunit of protein kinase A (74,80-94).

Presently, how these protein-protein interactions relate to the tumor suppressor activity of merlin is largely not understood. The association of merlin with CD44 and  $\beta$ 1-integrin raises the possibility that merlin might function as a molecular switch in the signaling pathways. CD44 is a transmembrane hyaluronic acid receptor implicated in cell-cell adhesion, cell-matrix adhesion, cell motility, and metastasis (93,94). Recent evidence suggests that merlin mediates contact inhibition of cell growth through signals from the extracellular matrix. At high cell density, merlin becomes hypo-phosphorylated and inhibits cell growth in response to hyaluronate, a mucopolysaccharide that surrounds cells. Merlin's growth-inhibitory activity depends on specific interaction with the cytoplasmic tail of CD44. At low cell density, merlin is phosphorylated, growth permissive, and exists in a complex with ezrin, moesin, and CD44. These data indicate that merlin and CD44 form a molecular switch that specifies cell growth arrest or proliferation (95). Rac1, a member of the Rho GTPase family, has recently been demonstrated to promote phosphorylation of merlin thereby inactivating its growth suppressor mechanism. Additionally, among the Rac/Cdc42 effectors, p21-activated kinase 2 (Pak2) has been shown to phosphorylate merlin at serine 518 and inactivates its function (79,96,97). Kissil et al. also recently reported an interaction between merlin and Pak1. Merlin inhibits the activation dynamic of Pak1. Loss of merlin expression leads to the inappropriate activation of Pak1, while overexpression of merlin results in the inhibition of Pak1 activity (82). (**Figure II**)

### **Merlin's growth regulatory function is related to its conformation and protein-protein interactions**

The activities of the ERM proteins are controlled by self-association of the proteins' N-terminal and C-terminal regions (98,99). The ERM proteins can exist in the 'closed' conformation, where the N- and C-terminal regions undergo an intramolecular interaction, thus folding the protein to mask the conserved actin-binding site (Figure III). The molecule can be converted to the 'open' conformation in which the intramolecular interaction is disrupted by signals such as phosphorylation or treatment with phosphoinositides (56,82,97,100).

Merlin's ability to function as a growth regulator is also related to its ability to form such intramolecular associations. Two such interactions have been identified. The first interaction is between residues which fold the N-terminal end of the protein onto itself, while the second interaction folds the entire protein so that there is contact between N and C-terminal ends of the protein (56,74,101,102) (**Figure II**). In a fashion similar to the ERM proteins, merlin may cycle between the 'open' and 'closed' conformations that differentially determine whether it binds with the ERM proteins or other molecules to transduce merlin's growth inhibition signal (103). In addition, the association between merlin and HRS, a substrate implicated in the signaling pathway initiated by hepatocyte growth factor (HGF) binding to the c-met receptor (104), appears to be regulated by merlin folding, suggesting that the ability of merlin to cycle between the 'open' and 'closed' conformations may integrate CD44 and HGF signaling pathways relevant to growth regulation (102).

### **The *NF2* gene promoter**

Another research focus, which is currently being investigated, is to characterize the upstream and downstream untranslated regions of the *NF2* gene so that these regions could be screened for mutations in both spontaneous and familial tumors in which no mutation was found in the *NF2*-coding region. We have mapped the major transcription initiation site of the *NF2* gene and found that multiple regions in the *NF2* promoter are required for full *NF2* promoter activity (64,105). Both positive and negative *cis*-acting regulatory elements required for transcription of the *NF2* gene have been found in the 5' flanking region of the promoter. A G/C-rich sequence located in the proximal promoter region, which can be bound by the Sp1 transcription factor, serves as a positive regulatory element. Both the 5' and 3' flanking regions of the human *NF2* locus are G/C rich and could serve as a target for gene methylation

and inactivation (64).

### **Alternatively spliced *NF2* mRNA isoforms in vestibular schwannomas and other cell types**

DNA consists of regions called exons and introns. The exons are the segments of DNA that are transcribed and brought together as a mature mRNA product. The introns represent the sections of DNA that are transcribed but are spliced out during RNA processing. Alternate splicing is the mechanism by which different exon combinations are brought together to produce multiple mRNA transcripts from the same gene. These alternately spliced transcripts can include all of the gene's exons, or can be missing one or multiple exons. The different RNA transcripts produced from this process are termed mRNA isoforms.

An example of *NF2* mRNA isoforms is shown in **Figure III**. The *NF2* gene undergoes alternative splicing in the coding exons. Multiple alternatively spliced *NF2* transcripts have been identified in various human cells. The most common isoforms in these cells were isoforms II (containing all 17 exons) and I (without exon 16) (33,64,106-111).

We have also examined the expression of alternatively spliced *NF2* mRNA isoforms in vestibular schwannomas (1 *NF2* schwannoma, 7 sporadic schwannomas, and 2 cystic schwannomas). Cloning and sequencing analysis showed that the expression pattern and relative frequency of the alternatively spliced *NF2* transcripts appeared to be different from those detected in other human cell types described above. Particularly, in addition to isoforms I and II, these schwannomas expressed a high percentage of the *NF2* mRNA isoform lacking exons 15 and 16. These alternatively spliced *NF2* transcripts could encode different protein products (our unpublished data).

Presently, the role of alternative splicing of *NF2* mRNA is not well understood. It is possible that the functional contribution of the *NF2* tumor suppressor may require a balanced expression of various isoform proteins in Schwann cells and/or other cell lineages (64,112). Alternatively, alternative splicing may be another mechanism for Schwann cells to inactivate merlin function and/or to generate isoforms that have additional properties conducive to tumor formation. We are presently conducting experiments to test these possibilities.

### **Immunohistochemical markers of growth in vestibular schwannomas: clinical correlation**

Attempts to correlate clinical parameters with immunohistologic evaluation of protein expression in vestibular schwannomas have been performed. An increase in Ki-67, which is an index of nuclear proliferation, was shown to correlate with the growth of solid schwannomas on MRI (113, 114). Higher rates of tumor recurrence have also been suggested in tumors with an increased rate of nuclear proliferation and mitotic indexes, although the supporting data for this claim is not conclusive (115). Positron emission tomography (PET) scanning has been conducted to assess the metabolic activity of vestibular schwannomas preoperatively and to correlate the metabolic activity with the proliferation index, Ki-67. No correlation was found between the large and recurrent tumors and the uptake of 18-fluorodeoxyglucose (FDG) as a radionuclide tracer to measure glucose metabolism by PET scanning. Additionally, there was no correlation between FDG uptake and Ki-67 expression measured by immunostaining (116). A possible reason for this is that vestibular schwannomas are slow growing tumors with only a small proportion of the tumor cells being in S-phase (active division) (117).

Another possible marker for tumor growth is the transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1). Immunostaining for TGF- $\beta$ 1 was positive in 96% of blood vessels within schwannomas and in 84% of schwannoma tissue samples; however again, no clinical correlation with tumor types or tumor growth was found (118). Immunohistochemical association of  $\beta$ 1-integrin with merlin has been demonstrated, but has not been related directly to tumor phenotypes (88). Cystic schwannomas are associated with a 36-fold decrease in nuclear proliferation as measured with Ki-67 staining when compared to solid tumors, suggesting that the rapid clinical growth seen in cystic schwannomas is related to the



accumulation of cyst formation but not by an actual increase in the growth rate of tumor cells (119,120). Also, NF2-associated schwannomas have been shown to have an increased proliferation index by Ki-67 and PCNA immunostaining, when compared to unilateral solid schwannomas (121,122).

Taken together, these studies demonstrate a degree of correlation between clinical growth as assessed by MRI scans and historical data, and nuclear growth indexes in solid unilateral and NF2-associated schwannomas. However, cystic tumor growth appears to occur via a different mechanism. Although the defective NF2 gene is the underlying common denominator in tumor formation of unilateral sporadic, NF2-associated, and cystic schwannomas, other differences at the molecular level likely account for the variable clinical presentations of these tumors.

### **Identifying deregulated genes in vestibular schwannomas**

With 69,227 mRNA sequences representing unique human genes and more than 3 million expressed sequence tags (ESTs) in the UniGene data base, the success of the Human Genome Project is evident. However, the expression, function, and regulation of the majority of genes are not yet known (123). The study of large-scale gene expression profiles utilizing cDNA microarray allows examination of the so-called 'transcriptome' of a tissue, and gives a means of exploring a broad view of the basic biology of tumors (124,125). Data from the human genome project makes the expression profiles more readily searchable, and organization of the genes into functional groupings allows examination of distinct pathways. For example, cell cycle control, DNA damage repair, or signal transduction and transcription factors can be organized and reviewed for various tumors (126). Biochips that contain thousands of oligonucleotides representing genes from the human genome have been created and are used to perform cDNA microarrays.

To evaluate the gene expression profile in a tumor, RNA is isolated from the tumor and converted into cDNA or cRNA. This cDNA or cRNA is then labeled with a fluorescent dye and hybridized to the oligonucleotides on the biochip. The same process can be used to evaluate RNA expressed in a normal tissue and compare gene expression differences between the diseased and normal tissues. Consequently, this helps scientists identify deregulated genes in the diseased tissue. Microarray gene expression analysis has been successfully utilized in recent years to evaluate a number of solid tumors including breast carcinoma, colon carcinoma, prostate carcinoma, ovarian carcinoma, and vestibular schwannomas (127-134).

Gene expression analysis has revealed differences among tumors which are not distinguishable histologically. Molecular classification, rather than histological classification, may also better predict the response of certain tumor types to specific therapies (135). This genomic scale approach has helped to identify sub-classes of colon carcinoma, breast carcinoma, melanoma, leukemia and lymphomas (132-134). In several instances, cDNA microarray analysis has already identified genes that appear to be useful for predicting clinical behavior.

Vestibular schwannoma characteristics can not be explained by the current understanding of the mutation types alone. Investigating inter-tumor variability of gene expression profiles shows promise to help unravel the clinical differences among subtypes of vestibular schwannomas. To better understand the pathways leading to schwannoma formation, we have utilized cDNA microarray analysis to evaluate gene expression profiles of vestibular schwannomas (127,128). Three sporadic vestibular schwannomas, two NF2-associated schwannomas, and three cystic schwannomas were compared to a normal vestibular nerve from a patient with a sporadic schwannoma. The goal was to seek patterns of gene expression consistently elevated or decreased across all tumors. Of 25,920 genes or ESTs screened, 42 genes were significantly up-regulated (by a factor of three or more) consistently across at least 6 of the 8 tumors examined. Additionally, multiple genes were found to be significantly down-regulated in the majority of vestibular schwannomas examined. Of these genes, eight genes involved with cell signaling and division were down-regulated, including an apoptosis-related, putative tumor suppressor

gene LUCA-15 which was down-regulated in 7 of 8 schwannomas studied.

Interesting and potentially important pathways for tumorigenesis are suggested by the deregulated genes. Two mediators of angiogenesis, endoglin and osteonectin, were highly elevated in most, if not all, tumors examined (127). Endoglin is a transforming growth factor- $\alpha$  receptor that is known to be an endothelial marker for angiogenesis in solid tumors, and osteonectin is a secreted glycoprotein that interacts with extracellular matrix proteins to decrease adhesion of cells from the matrix, thereby inducing a biological state conducive to cell migration. Endoglin was found to be significantly up-regulated in all of the solid tumors but not in any of the cystic tumors examined. The difference in endoglin gene expression may be a key to unlocking why some schwannomas develop into the aggressive cystic phenotype. Osteonectin was elevated in all of the tumors studied and may be a target for potential therapies including angiogenesis inhibitors (127,128,136,137). An example of a deregulated signaling pathway suggested by the microarray data is the retinoblastoma protein (pRb)-cyclin-dependent kinase (CDK) pathway (128). Among genes involved in G<sub>1</sub>-S progression, CDK2 was found to be down-regulated in 7 of 8 tumors. In addition, up-regulation of transforming factor RhoB was found in all of the schwannomas examined (127).

## Summary

The discovery of molecular mechanisms underlying vestibular schwannoma formation is rapidly moving forward. Understanding merlin's interactions with other proteins, signaling pathways, and regulation of the *NF2* gene will hopefully lead to the development of novel treatments for vestibular schwannomas. These treatments may not be as far in the future as one might think. For example, upregulation of the RhoB GTPase seen in vestibular schwannomas may respond to target specific therapy with Rho B inhibitors (138). Ultimately, drug therapies will be designed to stop schwannoma progression. This will offer alternatives to the current options of untreated observation of tumor growth, stereotactic radiation, or surgical removal. Furthermore, it may also be possible to develop targeted therapy that may shrink or altogether eradicate preexisting tumors. These are the challenges facing the "molecular neurotologist" of the future.

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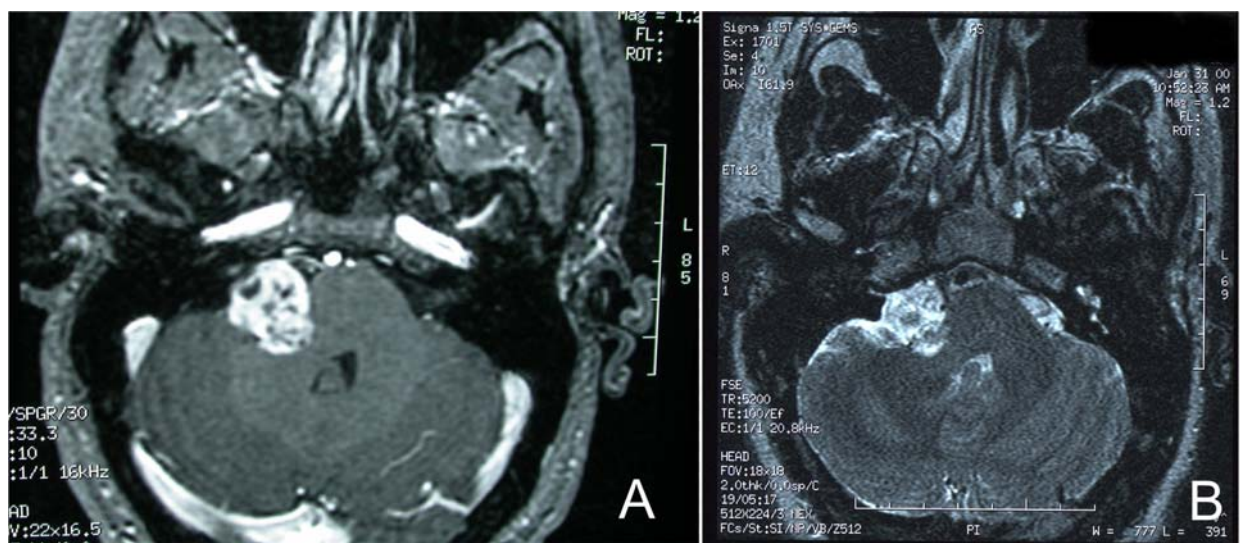
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**Figure 1. MRI images of vestibular schwannomas.** A) Axial T1 weighted MRI with gadolinium contrast. There is an enhancing right-sided cerebellopontine angle tumor with areas of central low intensity that correspond with cysts within this pathologically confirmed vestibular schwannoma. B) Axial T2 weighted MRI. The tumor is more hyperintense than the typical T2 signal characteristics of a vestibular schwannoma. Additionally, there are focal areas of increased signal intensity that correspond with the intra-tumoral cysts.



**Figure 2: Schematic diagram of merlin action.** This diagram shows how Rac1 and Pak help convert the merlin protein from a closed conformation to an open conformation by phosphorylation of the protein. Consequently, merlin, in its open conformation, can interact with CD44 and facilitate linking the actin cytoskeleton to the cell membrane.

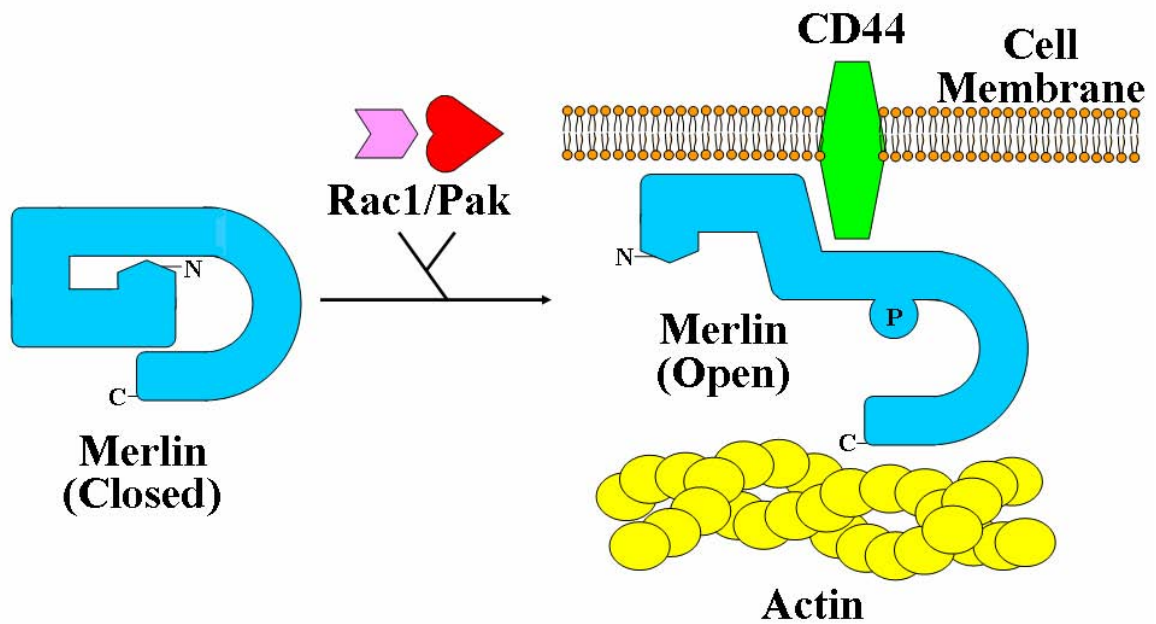
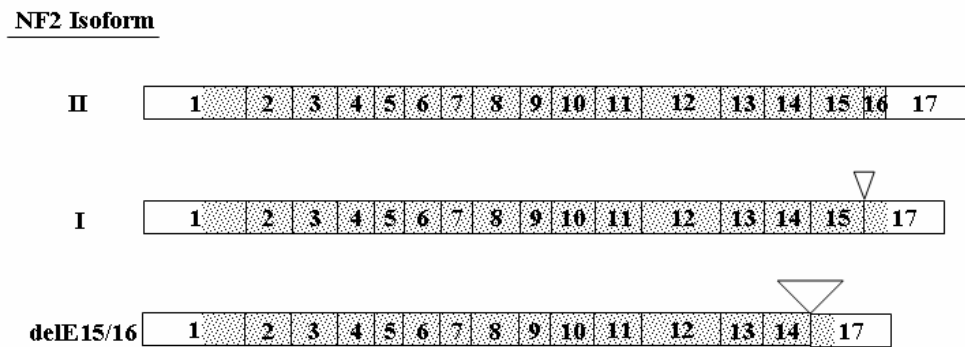


Figure 3: The *NF2* gene is transcribed into mRNA; however, alternative splicing can produce different mRNA transcripts or isoforms. Different exon combinations can be brought together to produce multiple mRNAs from the same gene. Our studies showed that isoform I, II and delE15/16 were the most common isoforms found in vestibular schwannomas examined.





# Regulation of the Neurofibromatosis 2 Gene Promoter Expression during Embryonic Development

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Keywords:	Neurofibromatosis 2 (NF2) gene promoter, neurofibromatosis type 2 (NF2), neural tube closure, neural crest cell migration, pigmented epithelium of the retina, transgenic mice

# Regulation of the *Neurofibromatosis 2* Gene Promoter Expression during Embryonic Development

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**ABSTRACT**

Mutations in the *Neurofibromatosis 2* (*NF2*) gene are associated with predisposition to vestibular schwannomas, spinal schwannomas, meningiomas, and ependymomas. Presently, how *NF2* is expressed during embryonic development and in the tissues affected by neurofibromatosis type 2 (*NF2*) has not been well-defined. To examine *NF2* expression *in vivo*, we generated transgenic mice carrying a 2.4-kb *NF2* promoter driving  $\beta$ -galactosidase ( $\beta$ -gal) with a nuclear localization signal. Whole-mount embryo staining revealed that the *NF2* promoter directed  $\beta$ -gal expression as early as embryonic day E5.5. Strong expression was detected at E6.5 in the embryonic ectoderm containing many mitotic cells.  $\beta$ -gal staining was also found in parts of embryonic endoderm and mesoderm. The  $\beta$ -gal staining pattern in the embryonic tissues was corroborated by *in situ* hybridization analysis of endogenous *Nf2* RNA expression. Importantly, we observed strong *NF2* promoter activity in the developing brain and in sites containing migrating cells including the neural tube closure, branchial arches, dorsal aorta, and paraaortic splanchnopleura. Furthermore, we noted a transient change of *NF2* promoter activity during neural crest cell migration. While little  $\beta$ -gal activity was detected in premigratory neural crest cells at the dorsal ridge region of the neural fold, significant activity was seen in the neural crest cells already migrating away from the dorsal neural tube. In addition, we detected considerable *NF2* promoter activity in various *NF2*-affected tissues such as acoustic ganglion, trigeminal ganglion, spinal ganglia, optic chiasma, the ependymal cell-containing tela choroidea, and the pigmented epithelium of the retina. The *NF2* promoter expression pattern during embryogenesis suggests a specific regulation of the *NF2* gene during neural crest cell migration and further support the role of merlin in cell adhesion, motility, and proliferation during development.

## INTRODUCTION

Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder that predisposes affected individuals to bilateral vestibular schwannomas and the development of multiple meningiomas, intracranial tumors, ophthalmologic and skin abnormalities, and spinal schwannomas (NIH Consens. Statement, 1991). By positional cloning, the gene associated with NF2 has been identified and termed the *Neurofibromatosis 2* gene (*NF2*), which encodes a protein named ‘merlin’ for moesin-ezrin-radixin like protein (Trofatter et al., 1993), or ‘schwannomin’, a word derived from schwannoma, the most prevalent tumor seen in NF2 (Rouleau et al., 1993). Mutations in the *NF2* gene have been found in NF2-associated vestibular schwannomas, sporadic vestibular schwannomas, and cystic schwannomas, as well as meningiomas (reviewed in Neff et al., 2005).

The *NF2* protein shares a high degree of homology to ezrin, radixin, and moesin (ERM), a family of membrane-cytoskeleton-associated proteins that are important for cell adhesion, motility, regulation of cell shape, and signal transduction (McClatchey 2004; McClatchey and Giovannini, 2005). Like the ERM proteins, merlin is expressed in a variety of cell types where it localizes to areas of membrane remodeling, particularly membrane ruffles, although its precise distribution may differ from the ERM proteins expressed in the same cell (Gonzalez-Agosti et al., 1996). In addition, schwannoma cells from NF2-associated tumors have dramatic alterations in the actin cytoskeleton and display abnormalities in cell spreading (Pelton et al., 1998). These results suggest that merlin may play an important role in regulating both actin cytoskeleton-mediated processes and cell proliferation. However, unlike the ERM proteins, merlin exerts a growth suppression effect. Over-expression of merlin in mouse fibroblasts or rat schwannoma cells can limit cell growth (Lutchman and Rouleau, 1995; Sherman et al., 1997; Gutmann et al.,

1998) and suppress transformation by a *ras* oncogene (Tikoo et al., 1994). Recent studies demonstrate that cells lacking *NF2* function exhibit characteristics of cells expressing activated alleles of the small GTPase Rac, and the p21-activated kinase 2, a downstream target of Rac1/Cdc42, which directly phosphorylates merlin, affecting merlin's localization and function (Shaw et al., 2001; Xiao et al., 2002; Kissil et al., 2002; Surace et al., 2004; Rong et al., 2004).

Studies of *Nf2* gene knockout in mice show that merlin function is essential during early embryonic development. Homozygous *Nf2* mutant mouse embryos fail in development at approximately day 7 of gestation and die immediately prior to gastrulation (McClatchey et al., 1997). Conditional homozygous deletion of *Nf2* in Schwann cells or arachnoid cells leads to hyperplasia and tumor development, which are characteristics of NF2 (Giovannini et al., 2000; Kalamarides et al., 2002). Although these results argue that loss of merlin is sufficient for schwannoma and meningioma formation *in vivo*, none of the lesions detected in these mice was found in the vestibular nerve. This observation contrasts with the vestibular schwannomas commonly found in patients with NF2.

To better understand merlin function during development, previous studies examined merlin expression using Northern blot, *in situ* hybridization, RT-PCR, or immunostaining; however, these studies have not yielded consistent results. An earlier report indicated that the *NF2* gene was only expressed in tissues of ectodermal origin (World Health Org., 1992). Subsequently, Bianchi et al. (1994) reported that merlin RNA was not detected in the adult human heart and liver, whereas Haase et al. (1994) noticed abundant merlin RNA expression in the adult mouse heart. Similarly, no merlin RNA was detected in the adult mouse lung, whereas abundant expression could be found in the adult human lung. Northern blot analysis, however, detected *Nf2* transcripts in the adult mouse brain, kidney, cardiac muscle, skin and lung (Claudio



et al., 1994). By *in situ* hybridization and reverse transcription-polymerase chain reaction (RT-PCR) analyses, Gutmann et al. (1994) reported that rat merlin was widely expressed during mid to late embryogenesis. High levels of merlin expression were seen in cerebral cortex, brainstem, spinal cord, and heart during embryonic days E12~16. Merlin RNA expression becomes restricted to the brainstem, cerebellum, dorsal root ganglia, spinal cord, adrenal gland and testis in adult animals, while no appreciable levels of merlin RNA could be detected in kidney, lung, and skeletal muscle. On the contrary, by *in situ* hybridization and immunostaining, Huynh et al. (1996) showed that merlin was detected in most differentiated tissues but not in undifferentiated tissues. In particular, merlin was not detectable in mitotic neuroepithelial cells, the perichondrium, the liver, the neocortex, and the ventricular zone of the developing cerebral cortex. Furthermore, in contrast to the phenotype of early embryonic lethality in mice lacking *Nf2* function, Gronholm et al. (2005) did not detect merlin protein expression until E11 in mouse embryos. In light of these inconsistent results, a detailed analysis of *NF2* expression during embryonic development is needed.

Previously we have defined the 5' flanking sequence of the human *NF2* gene and showed that the 2.4-kb *NF2* promoter could direct strong expression in several cell lines including neuronal cells (Welling et al., 2000; Chang et al., 2002). However, whether the *NF2* promoter is sufficient for expression in a variety of tissues including Schwann cells and neurons *in vivo* has not been tested. The objective of this study was to define the *NF2* expression pattern during embryonic development using two approaches. First, we generated a construct containing the 2.4-kb *NF2* promoter-driven  $\beta$ -galactosidase ( $\beta$ -gal) with a nuclear localization signal and used it to produce transgenic mice. Whole-mount X-gal staining of transgenic embryos at various days post coitus (p.c.) was conducted and tissue sections were analyzed. Second, we performed

whole-mount *in situ* hybridization using various *Nf2* cDNA fragments as probes to confirm the expression pattern. Our results show that the *NF2* promoter could direct  $\beta$ -gal expression as early as E5.5.  $\beta$ -gal expression was first detected in the embryonic ectoderm and to a lesser extent, in some parts of endoderm and mesoderm. Subsequently, strong  $\beta$ -gal staining was seen in the developing neural tube, migrating neural crest cells, the heart, the dorsal aorta, and the paraaortic mesenchyme. As the embryos matured, significant levels of  $\beta$ -gal expression were found in the cranial ganglia V and VIII, spinal ganglia, pigmented epithelium of the retina, and skin.

## MATERIALS AND METHODS

*Transgene construct and transgenic production.* The pNF2P(-2092)-Luc plasmid containing the 2.4-kb human *NF2* promoter was described previously (Chang et al., 2002). The MFG-S-nlsLacZ retroviral vector was kindly provided by Dr. Bruce Bunnell (Imbert et al., 1998). To generate the pNF2P(-2092)-nlsLacZ construct, the luciferase expression unit was removed from pNF2P(-2092)-Luc and substituted with the LacZ gene, which contained a nuclear localization signal (nlsLacZ) and was excised from MFG-S-nlsLacZ vector. Subsequently, the splicing signal and the polyadenylation signal sequences isolated from pSV2- $\beta$ G (Chang et al., 1989) were inserted downstream of the nlsLacZ sequence (Figure 1A).

The *NF2* promoter-driven nls-*LacZ* expression cassette was excised from the pNF2P2.4-nls-LacZ plasmid by double digestions with *NotI* and *SalI* enzymes. The *NF2* promoter-nls-LacZ DNA fragment was purified through a Qiaquick Gel Extraction kit (Qiagen) and microinjected into male pronuclei of fertilized one-cell mouse eggs obtained from superovulated FVB/N female mice (Hogan et al., 1994). Injected embryos were transferred into the oviduct of

pseudopregnant female foster mice to allow complete development to term.

To identify transgenic mice, mouse-tail DNA was prepared using the Puregene kit (Gentra) and used in Southern blot analysis. High-molecular-weight mouse-tail DNA was digested with *Bam*HI enzyme, which cut once between the *NF2* promoter and nls-*LacZ* DNA of the transgene. Digested DNA was electrophoresed onto a 0.7% agarose gel and then transferred to a GeneScreen Plus<sup>®</sup> hybridization transfer membrane (NEN Life Science). For the probe, the *LacZ* DNA was labeled with biotinylated dNTP mixture by the random primed method using the NEBlot<sup>™</sup> Phototope<sup>™</sup> kit (New England Biolabs). Filter membranes containing mouse-tail DNAs were prehybridized in hybridization buffer for one hour, and then hybridized with the biotin-labeled *LacZ* probe overnight. After hybridization, filters were washed twice in 0.1X SSC and 0.1% SDS at 65°C for 30 min each time. For detecting hybridization signal, the Phototope<sup>™</sup>-Star Detection Kit for Nucleic Acids (New England Biolabs) was used, and chemiluminescence was captured by the ChemiGenius<sup>2</sup> Image Acquisition System (Syngene) or by exposure to X-ray films. Once identified, transgenic mice were mated with FVB/N mice to generate offspring.

*Whole-mount X-gal staining.* Transgenic mice were mated with each other. The day when the vaginal plug was found, the embryo was aged as 0.5 day p.c. (E0.5). On the following day the embryo was aged as E1.5 and so on. Embryos at various days p.c. were harvested and fixed in the fixative solution containing 1% formaldehyde, 0.2% glutaraldehyde, and 0.02% NP-40 in phosphate-buffered saline (PBS) for 40 min on ice. After fixation, embryos were incubated overnight in the X-gal staining solution, containing 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 2 mM MgCl<sub>2</sub>, and 1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-galactoside, at 37°C with gentle shaking.

Stained embryos were rinsed with PBS and photographed under a Leica MZ16FA stereoscope. Embryos were further fixed overnight in 4% paraformaldehyde in PBS at 4°C and then embedded in paraffin. Five-micron tissue sections were obtained using a rotary microtome. Sections were deparaffinized, counter-stained with nuclear fast red, mounted with a coverslip, and then photographed under a Leica DM4000B microscope.

*Cloning of mouse Nf2 cDNAs.* Total RNA was isolated from adult mouse brain using the TRIzol reagent (Invitrogen) and used in RT-PCR to isolate *Nf2* cDNAs (Figure 1B) as described previously (Chang et al., 2002). The mouse *Nf2* cDNA containing the entire coding region was obtained by RT-PCR using the primers Coding-F and Coding-R (Figure 1C). The resulting *Nf2* cDNA was cloned into pCRII-TOPO vector (Invitrogen) to generate the pCRII-*Nf2* coding plasmid (Figure 1B). The *Nf2* cDNA was digested with *HindIII* enzyme to yield the N-terminal 0.9-kb, middle 0.3-kb, and C-terminal 0.6-kb fragments. Each cDNA fragment was subcloned into pCRII-TOPO to generate the pCRII-N, pCRII-M, or pCRII-C subclone, respectively. To obtain the *Nf2* cDNA containing the 5' untranslated region, RT-PCR was performed using the 5U-F and 5U-R primers (Figure 1C). The resulting cDNA product was cloned into pCRII-TOPO to generate the pCRII-5U construct (Figure 1B). Similarly, cDNAs containing the sequences immediately upstream of the translation termination codon and extending into the 3' untranslated region were obtained using the primer pairs 3U1-F and 3U1-R, or 3U2-F and 3U2-R (Figure 1C). The resulting cDNAs containing the 3' untranslated region were also cloned into pCRII-TOPO to generate pCRII-3U1 and pCRII-3U2, respectively (Figure 1B). All *Nf2* cDNA sequences obtained were confirmed by DNA sequencing.

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*Whole-Mount RNA In situ hybridization.* Mouse embryos (E7.5, E8.5, and E9.5) were harvested and fixed in 4% paraformaldehyde in PBS overnight at 4°C. Fixed embryos were rinsed with PBT (PBS plus 0.1% Tween) three times, placed in 100% methanol, and then bleached at room temperature for five hours by adding hydrogen peroxide to 6%. After rinsing with 100% methanol three times, embryos were stored in 100% methanol at -20°C.

*In situ* hybridization was performed as previously described (Wilkinson, 1992) with minor modifications (Correia and Conlon, 2001). Following hydration through a 75%, 50%, 25% methanol/PBT series, embryos were treated with 10 mg/ml proteinase K in PBT at room temperature (five min for E7.5 embryos, seven min for E8.5 embryos, and eight min for E9.5 embryos). Treated embryos were washed twice for five min with 2 mg/ml glycine in PBT, rinsed three times with PBT, and then re-fixed with freshly prepared 4% paraformaldehyde/0.2% glutaraldehyde in PBT for 20 min at room temperature.

For riboprobe preparation, transcription plasmids carrying different portion of the *Nf2* cDNA (pCRII-5U, N, M, C, 3U1, and 3U2) were linearized with an appropriate restriction enzyme, which cuts at the junction between the cDNA and vector sequences. *In vitro* transcription that produced riboprobes, which incorporate digoxigenin-labeled nucleotides from each linearized plasmid with T7 or SP6 polymerase was performed using the DIG RNA Labeling Kit (Roche). Both the sense and antisense riboprobes from each transcription plasmid were produced.

For hybridization, embryos were briefly rinsed with hybridization buffer (5X SSC, pH 5, 1% SDS, 50 µg/ml yeast tRNA, 50 µg/ml heparin, and 50% formamide) and then incubated in hybridization buffer for 1 hour at 65°C with gentle shaking. After removing the pre-hybridization buffer, each riboprobe was diluted in hybridization buffer to about 1 µg/ml and

then added to the embryos. Hybridization was carried out at 65°C with gentle shaking overnight.

Hybridized embryos were sequentially washed with Wash Solution 1, a 1:1 solution of Wash Solutions 1 and 2, and Wash Solution 2, followed by digestion with 100 µg/ml RNase A and washing with Wash Solution 2 and 3 (Wilkinson, 1992). To detect the hybridization signal, the DIG Nucleic Acid Detection Kit (Roche) was used. Embryos were pre-blocked with 10% sheep serum and then incubated overnight at 4°C with alkaline phosphatase-conjugated anti-digoxigenin antibody, which had been pre-absorbed with embryo powder (Wilkinson, 1992). After extensive washing, embryos were incubated in 1 ml of freshly prepared NTMT (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM MgCl<sub>2</sub>, and 0.1% Tween 20) containing 4.5 µl/ml NBT stock and 3.5 µl/ml BCIP stock (Correia and Conlon, 2001). Incubation was performed in the dark with gentle shaking. When color was developed to the desired extent, embryos were rinsed several times with PBT, stored in a 50/50 mix of glycerol and PBT, and photographed under a Leica MZ16FA stereoscope.

*Immunohistochemical analysis.* Embryos at various days p.c. were harvested, fixed in 4% paraformaldehyde, and then embedded in paraffin. Tissue sections were obtained, deparaffinized and processed for immunostaining with antibodies against Sox 9 (sc-20095; Santa Cruz Biotechnology), S100 (z 0311; Dako), and merlin (sc-331; Santa Cruz Biotechnology) according to previously described procedures (Welling et al., 2002). Negative controls were treated with the same immunostaining procedure except without the primary antibody. Hematoxylin was used as a counterstain.

## RESULTS

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*The NF2 promoter directed transgene expression as early as E5.5.* To examine the *NF2* promoter expression pattern *in vivo*, we generated the pNF2P2.4-n $\beta$ gal construct containing the  $\beta$ -gal reporter with a nuclear localization signal under the control of the 2.4-kb human *NF2* promoter (Figure 1A), and used it to produce transgenic mice. Four lines of transgenic NF2P2.4-n $\beta$ gal mice were generated. To detect the transgene-encoded  $\beta$ -gal, embryos were obtained from the mating of all four lines of transgenic mice at various days p.c. and whole-mount X-gal staining was performed. All four lines of the transgenic NF2P2.4-n $\beta$ gal mice showed a similar  $\beta$ -gal staining pattern, eliminating the positional effect due to integration.

As shown in Figure 2A,  $\beta$ -gal staining could be seen in the transgenic embryo as early as E5.5. At this stage,  $\beta$ -gal expression was detected only in the embryonic tissue but not in the extraembryonic tissue. At E6.5, strong  $\beta$ -gal staining was found in the embryonic ectoderm. Cells in this embryonic tissue divided rapidly with visible mitotic figures, and were darkly stained (Figure 2B). Significant  $\beta$ -gal expression was also seen in some parts of the proximal embryonic endoderm. Weak  $\beta$ -gal staining was detected in the mesoderm, while no staining was seen in the extraembryonic ectoderm (Figure 2A). E7.5 is the stage, when a portion of the dorsal embryonic ectoderm begins to specify into the neural ectoderm, a process important to the formation and shaping of the neural plate (Hogan et al., 1994; Rugh, 1994). High levels of  $\beta$ -gal expression continued to be observed in the embryonic ectoderm of the transgenic E7.5 embryo (Figure 2A). Similar to that observed at E6.5, substantial  $\beta$ -gal staining was also detected in the embryonic endoderm. Intriguingly, the staining was not contiguous in this endoderm at E7.5; some regions were extensively labeled while others were not. In the mesoderm, only a few cells showed significant  $\beta$ -gal staining, while in the extraembryonic tissues, the ectoplacental cone and the chorionic ectoderm remained negative for  $\beta$ -gal expression.



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*The transgene-encoded  $\beta$ -gal staining pattern coincided with the endogenous *Nf2* RNA expression pattern in the embryonic tissues.* As mentioned earlier, previous studies examining merlin expression, particularly using *in situ* hybridization and immunostaining (Gutmann et al., 1994; Gutmann et al., 1996), did not yield consistent results. To examine whether the 2.4-kb *NF2* promoter could recapitulate the endogenous *Nf2* expression pattern, we performed whole-mount RNA *in situ* hybridization analysis. Various regions of the *Nf2* cDNA were cloned into the pCRII vector (Figure 1B). Both sense and antisense riboprobes were synthesized from each plasmid by *in vitro* transcription and used in whole-mount embryo hybridization. We found that the antisense probe prepared from 3U1, containing the *Nf2* sequence immediately upstream of the translation termination codon to about 300 bp into the 3' untranslated region (Figure 1), consistently gave rise to a lower background when the sense probe was compared with the antisense probe. The representative images of whole-mount RNA *in situ* hybridization of E7.5 embryos were shown in Figure 3. *Nf2* RNA expression was readily detected throughout the embedded embryo and its surrounding decidua, when the antisense probe, derived from 3U1, was used. In contrast, the sense probe yielded little hybridization (compare Figure 3A with Figure 3B). To visualize what embryonic tissues expressed *Nf2* RNA, dissected embryos were used in the *in situ* hybridization experiment. High levels of *Nf2* RNA expression were detected in the embryonic tissues, particularly in the developing neural ectoderm (Figure 3C). For comparison, we performed whole-mount X-gal staining of transgenic E7.5 embryos. As shown in Figure 3D, the  $\beta$ -gal staining pattern in the embryonic tissue was similar to the endogenous *Nf2* RNA expression pattern, exhibiting the strongest staining in the neural ectoderm.

In addition, we performed whole-mount RNA *in situ* hybridization and  $\beta$ -gal staining of



E8.5 and E9.5 embryos. Similar to that observed in the E7.5 embryo, *Nf2* RNA expression was detected throughout the E8.5 embryo with the strongest expression in the developing neural tube (compared Figure 4A with Figure 4B). Also, *Nf2* RNA expression was detected in the allantois and the yolk sac (Figure 4A). Consistent with the RNA *in situ* hybridization result, strong  $\beta$ -gal staining was seen in the embryonic tissues of transgenic E8.5 embryo, particularly in the neural tube (Figure 4C), while no  $\beta$ -gal staining was found in the wild-type E8.5 embryo (Figure 4D). It should be noted that at this stage,  $\beta$ -gal expression was detected in the allantois and the yolk sac, but was not seen in the ectoplacental cone (Figure 4C). As the embryo matured to E9.5, *Nf2* RNA expression was consistently detected throughout the entire embryo with the strongest expression in the developing brain and spinal cord (compare Figure 5A with Figure 5B). The neural crest cell-populated branchial arches and the hematopoietic stem cell-containing paraaortic splanchnopleura also showed significant *Nf2* RNA expression (Figure 5A). A similar  $\beta$ -gal staining pattern was detected in the transgenic E9.5 embryo (Figure 5C). The tissues that gave rise to the strongest  $\beta$ -gal staining included the brain, spinal cord, and heart regions, the branchial arches, and the paraaortic splanchnopleura along with the dorsal aorta. Taken together, these results indicate that the  $\beta$ -gal staining pattern qualitatively matches most of the *Nf2* RNA distribution pattern, particularly in the embryonic tissues.

*Changes in NF2 promoter activity during neural crest cell migration.* Around E8.5 which is the early stage of organogenesis, the neural ectoderm-derived neural plate folds into the neural tube. Examination of  $\beta$ -gal expressing cells in the E8.5 transgenic embryo section detected the highest level of expression in the neural tube, particularly in the rostral end, and the intensity of the  $\beta$ -gal staining gradually decreased toward the caudal extremity (Figure 4E). Intriguingly,

cells in the dorsal ridge of the neural folds in the cranial region and its adjacent non-neural ectoderm were only modestly stained (arrowheads pointed to this region in Figure 4E). Previous studies have shown that the neural crest cells arise in the neural folds at the border between the neural and non-neural ectoderm (Hogan et al., 1994; LeDouarin and Kalcheim, 1999). Although initially contained within the central nervous system, the neural crest cells depart from the site of origin, migrate extensively throughout the embryo, and form many diverse derivatives including most of the peripheral nervous system, facial skeleton, and melanocytes of the skin. A detailed analysis of transversal sections of the anterior neural tube from transgenic embryos at around E8.5 revealed that while  $\beta$ -gal staining was detected in the neural folds, little staining was seen in the round-shaped neural crest cells which were in the process of delaminating from the dorsal ridge region of the neural fold (Figure 4F). However, significant  $\beta$ -gal staining was detected in the neural crest cells already migrating away from the dorsal neural tube (Figure 4G). Blue stained cells were detected along the putative pathways of neural crest cell migration particularly in the dorsal trunk mesenchyme beneath the ectoderm and between the somite and neural tube (Figures 4E and G; also see below). A number of markers on neural crest cells have been used to trace their migration. Among them, the Sox9 transcription factor is important for neural crest induction, survival, and delamination (Cheung and Briscoe, 2003; Mori-Akiyama et al., 2003). Interestingly, we observed abundant Sox9 protein expression in the migrating neural crest cells (Figures 6A and B). Substantial  $\beta$ -gal expression was observed in the endocardium of the heart (Figure 4E) and within the wall of the dorsal aorta (Figure 4G).  $\beta$ -gal staining was also found in the yolk sac and allantois (Figure 4E). At this embryonic stage, the yolk sac consists of an endodermal epithelium and underlying mesoderm within which blood islands and vessels develop. Significant  $\beta$ -gal expression was detected in the endodermal epithelium of the yolk sac

and some labeled cells were seen within the blood island.

Upon examination of tissue sections of the transgenic E9.5 embryos, highly labeled cells continued to be detected in the neural tube. Within the neural tube, high levels of  $\beta$ -gal activity were found in the developing forebrain, midbrain, and hindbrain (Figures 5C and 5D). As reported previously (LeDouarin and Kalcheim, 1999), the neural crest-derived cells from the posterior midbrain and hindbrain region migrate ventrolaterally and densely populate the first, second, and third branchial arches. Significant  $\beta$ -gal expression was seen in the cells of the craniofacial mesenchyme and the first branchial arch in the pharyngeal region (Figure 5D). The entire mesenchymal component of the branchial arch, which was derived from the neural crest cells, was highly labeled, whereas the epithelium covering the branchial arch and the foregut endoderm were not labeled. Strong  $\beta$ -gal staining was also detected in the paraaortic splanchnopleura, and the heart region and the dorsal aorta were also positive for  $\beta$ -gal staining (Figure 5D).

*The most intense  $\beta$ -gal staining was detected along the dorsal midline of the neural tube.* Whole-mount embryo staining showed that the *NF2* promoter-directed  $\beta$ -gal expression was predominantly observed in the anterior part of the transgenic embryo at E9.5 (Figure 7A). The  $\beta$ -gal staining extended to the posterior extremity as the embryo matured from E10.5 to E14.5 (Figures 7B, 7C, and 7D). By E14.5, extensive  $\beta$ -gal expression was detected throughout the embryo (Figure 7D).

Previous studies (Rugh, 1994; Wallingord, 2005) indicate that the neural tube begins to close at E8.5 from multiple sites in the middle portion of the embryo and extends toward the anterior and posterior ends in a zipper-like fashion. By E9.5, most parts of the neural tube have

already closed, and only small openings, called neural pores, are left in both the anterior and posterior ends of the embryo. We found that the most intense  $\beta$ -gal staining was located along the dorsal midline, the line of the neural tube closure, in the E9.5 embryo (Figure 8A). Deep staining was observed particularly in the area of the anterior neuropore, forming the fourth brain ventricle, also known as myelocoel. By E10.5, the anterior neuropore is completely closed (Rugh, 1994; Wallingord, 2005). Intense  $\beta$ -gal staining was still observed at the site of the thin roof of the fourth ventricle and along the dorsal midline of the neural tube (Figure 8B). Consistent with those observed at the earlier stages of development, strong  $\beta$ -gal expression was detected in the branchial arches I-IV of the E10.5 embryo (Figure 8C).

*Strong  $\beta$ -gal expression in the embryonic ectoderm-derived tissues.* In tissue sections of the E10.5 embryo, very intense  $\beta$ -gal labeling was noted in the tela choroidea, which is the thin roof of the fourth ventricle (Figure 9A). This roof plate consists of a single layer of ependymal cells, which is later covered by the pia mater, the inner layer of the meninges (Rugh, 1994). Significant  $\beta$ -gal expression was detected in the metencephalon and the myelencephalon. In addition, strong  $\beta$ -gal staining was found in the forebrain, including the telencephalon and the diencephalon, the optic chiasma, the tuberculum posterius, and the infundibulum (Figure 9B). However, only some parts of the mesencephalon were darkly stained while others were lightly stained. In the head region, the epidermal layer, which contains presumptive melanocytes, was also labeled (Figure 9A).

The retina is the innermost layer of the eye and is derived embryologically from the outgrowth of the developing brain (Martinez-Morales et al., 2004). It is comprised of two major layers, the inner layer (prospective neural layer of the retina) and the outer layer (prospective

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3 pigmented epithelium). In the E10.5 transgenic embryo, intense  $\beta$ -gal staining was readily  
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5 detected in the pigmented epithelium layer of the retina, whereas the neural layer of the retina  
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7 and the lens show very little expression (Figure 9C).  
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11 As noted above, strong  $\beta$ -gal activity was detected in the mesenchyme of the mandible  
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13 prominence of the first branchial arch in the E10.5 transgenic embryo (Figure 9D). The adjacent  
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15 neural crest cells populating the truncus arteriosus also showed intense labeling. In addition,  
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17 significant  $\beta$ -gal expression was found in the paraaortic mesenchyme and the heart region.  
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19 Furthermore, the dorsal aspect of the forming spinal cord and its flanking primordial spinal  
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21 ganglia were strongly labeled. Together, these results indicate that the *NF2* promoter is strongly  
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23 expressed in various embryonic ectoderm-derived tissues.  
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30 *NF2 promoter directed  $\beta$ -gal expression to the trigeminal ganglion and acoustic ganglion.*

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32 At E11.5, the forebrain is separated into a paired telencephalic vesicles and the diencephalon.  
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34 We observed high levels of  $\beta$ -gal activity in both lobes of the telencephaon and in the  
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36 diencephalon of the E11.5 transgenic embryo (Figure 10A). However, the midbrain  
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38 mesencephalon was only lightly stained with the exception of the dorsal midline closure, which  
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40 consistently displayed intense staining similar to those seen at earlier stages. Interestingly, we  
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42 detected a striped pattern of  $\beta$ -gal staining in the hindbrain-derived myelencephalon (Figure  
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44 10B) and the metencephalon (also see below).  $\beta$ -gal expression can also be found in the cranial  
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46 ganglion VIII, derived from the hindbrain and also known as the acoustic ganglion, and its  
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48 extending nerve. The  $\beta$ -gal staining was particularly notable in the cells surrounding the  
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50 acoustic ganglion and extending nerve (Figure 10B). It should be mentioned that the extending  
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52 nerve expressed higher level of S100 immunoreactivity than the ganglion (Figure 6C). Some of  
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the cells inside the ganglion also expressed  $\beta$ -gal (Figure 10B). In addition, strong  $\beta$ -gal staining was detected in the cranial ganglion V, which is also called the trigeminal ganglion; both the trigeminal ganglion and its three nerve divisions were robustly labeled (Figure 10C). Consistent with the  $\beta$ -gal staining, immunostaining revealed that merlin was expressed throughout the trigeminal ganglion (Figure 6D). Similar to that seen at E10.5, very intense  $\beta$ -gal staining was seen in the pigmented epithelium of the retina (Figure 10C).

As noted before, the dorsal midline of the spinal cord from the E11.5 embryo was darkly stained for  $\beta$ -gal expression; however, only a few cells inside the spinal cord were labeled (Figures 10D and 10E). Interestingly, we noted that the cells in the dorsal midline of neural tube closure expressed high level of Sox9, a neural crest determinant marker (Figure 6E).  $\beta$ -gal staining was detected in the cells surrounding the spinal ganglia and in some, but few, cells inside the spinal ganglia. It appeared that the cells along the ventral and dorsolateral pathways of neural crest cell migration were labeled (Figure 10E). The sclerotome of somites has been shown to play an essential role in neural crest migration of the early ventral pathway (Hogan et al., 1994; Chen et al., 2004; Hay 2005; Honjo and Eisen, 2005). Significantly, we also detected deep  $\beta$ -gal staining in the sclerotome (Figure 10F).

In addition to intense  $\beta$ -gal staining in the dorsal aorta as seen in earlier stages, the paraaortic mesenchyme were strongly labeled at E11.5 (Figure 10G). Within the four-chambered heart, the endometrial tissue, including the valves, showed the highest  $\beta$ -gal activity. Intervertebral arteries were also labeled. While some  $\beta$ -gal was expressed in the liver and mesonephros, only weak staining was detected in the gonad (Figures 10F and 10G).

*Broad  $\beta$ -gal expression pattern in various neural tissues during mid-embryogenesis. At*

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E12.5, the anterior portion of the telencephalon continued to express high levels of  $\beta$ -gal, while the staining in the posterior part was less saturated (Figure 11A). Similarly, some portions of the diencephalon expressed significant levels of  $\beta$ -gal, whereas other regions were weakly stained (Figure 11B). Curiously, the pigmented epithelium of the retina continued to be intensely labeled at this stage. The lens epithelium and some cells in the lens also expressed moderate levels of  $\beta$ -gal.

The posterior commissure is the roof of the brain between the anterior limit of the mesencephalon and the posterior portion of the diencephalon. Saturated  $\beta$ -gal staining was observed in the posterior commissure, while only patchy staining was seen in the rest of the mesencephalon (Figure 11C). However, it appeared that more labeled cells present in the mesencephalon proximal to the posterior commissure. The isthmus or mesencephalon-metencephalon junction tissue is an organizing center that plays an important role in the midbrain-hindbrain patterning (Wassef and Joyner, 1997). Intriguingly, intense  $\beta$ -gal staining was found in the isthmus tissue of the mesencephalic part (Figure 11D). In contrast, the metencephalic portion of this junction tissue showed a striped  $\beta$ -gal staining pattern, similar to that of the rest of the metencephalon. Consistent with robust  $\beta$ -gal expression in the tela choroidea observed at earlier stages, the tela choroidea-derived posterior choroid plexus was strongly labeled in the E12.5 embryo. The tuberculum posterius is a thickening in the floor of the brain at the region of the anterior end of the notochord. It represents the posterior margin of the diencephalon and develops into a part of the hypothalamus. We noted that some discrete regions of the tuberculum posterius showed high levels of  $\beta$ -gal activity, while some other parts expressed notably smaller amounts of  $\beta$ -gal (Figure 11E). At this stage, significant  $\beta$ -gal staining was still seen in the trigeminal ganglion and its nerve divisions; however, unlike the

strong labeling throughout the entire ganglion observed at E11.5, the central part of the trigeminal ganglion from the E12.5 embryo appeared to show little  $\beta$ -gal staining (Figure 11F). Interestingly, the synaptic junction area between the trigeminal ganglion and the hindbrain remained strongly labeled (Figures 10C and 11F). In addition, similar to that noted at E11.5, cells surrounding spinal ganglia and their extending nerves continued to show significant  $\beta$ -gal expression in the E12.5 embryo (Figure 11G). Taken together, these results indicate that the *NF2* promoter is widely expressed in neural tissues during embryogenesis.

## DISCUSSION

The development of schwannomas on or around the vestibular branch of both eighth cranial nerves has been considered as the hallmark of NF2, but other tumors and ocular abnormalities are observed as well (Neff et al., 2005). Most NF2 patients go on to develop multiple schwannomas that are associated with other cranial nerves, such as the trigeminal nerve and the spinal nerve roots. In addition, cranial and spinal meningiomas and, less frequently, ependymomas occur. Such restricted symptoms and phenotypes associated with NF2 are unusual, given the fact that the *NF2* tumor suppressor protein is widely expressed in many cell types. In this report, we showed that the *NF2* promoter was active at early embryogenesis. *NF2* promoter-directed  $\beta$ -gal expression was detected as early as E5.5 and intense  $\beta$ -gal staining was observed at E6.5 in the embryonic ectoderm containing many mitotic cells. In addition, *NF2* promoter activity was detected in parts of the embryonic endoderm and mesoderm. *NF2* promoter continued to be actively expressed in the neural ectoderm and its derived neural tissues throughout mid-embryogenesis. These results are consistent with earlier findings (Gutmann et al., 1994; McClatchey et al., 1997; Stemmer-Rachamimov et al., 1997) and further indicate that



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3 *NF2* is an early expression marker.  
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6 Currently, limited information is known about the role of merlin during embryonic  
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8 development and tissue differentiation. In mice, homozygous *Nf2* inactivation is embryonically  
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10 lethal (McClatchey et al., 1997). Although these results suggest an essential role for *NF2* during  
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12 early embryogenesis, the function of merlin in these processes is not understood. Merlin has  
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14 been shown to regulate cell motility and cell adhesion. In cultured mammalian cells, merlin is  
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16 concentrated in the membrane ruffle and adherens junction (Gonzalez-Agosti et al., 1996; Shaw  
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18 et al., 1998; Maeda et al., 1999; Lallemand et al., 2003). In cultured polarized neurons, merlin  
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20 localizes to synaptic junctions (Gronholm et al. 2005). Merlin can associate with the actin  
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22 cytoskeleton directly (Xu and Gutmann, 1998) or indirectly by interacting with actin-binding  
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24 proteins (Scoles et al., 1998; Fernandez-Valle et al., 2002). Re-expression of merlin in *Nf2*-  
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26 deficient cells attenuates actin cytoskeleton-associated processes, including motility (Gutmann et  
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28 al., 1999). In addition, over-expression of merlin mutants alters cell adhesion, causing  
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30 fibroblasts to detach from the substratum (Stokowski and Cox, 2000). Also, *Nf2* deficiency  
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32 results in an inability of mouse fibroblasts or keratinocytes to undergo contact-dependent growth  
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34 arrest and to form stable cadherin-containing cell:cell junctions (Lallemand et al., 2003). Merlin  
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36 may stabilize adherens junctions by inhibiting Rac/Pak signaling and stabilizing the actin  
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38 cytoskeleton (Shaw et al. 2001; Kissil et al., 2002; Xiao et al., 2002; McClatchey and  
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40 Giovannini, 2005). Moreover, *Nf2*-deficient mouse tumor cells are highly motile and metastatic  
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42 *in vivo* (McClatchey et al., 1998). Together, these results suggest that merlin may participate in  
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44 fundamental processes involving the regulation of cell migration, cell adhesion and cell  
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46 proliferation during embryonic development.  
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55 It has been well-documented that during embryogenesis, many cells and tissues undergo  
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complex morphogenetic movements, such as neural crest and progenitor germ cell migration, migration of hematopoietic progenitors into the embryonic hematopoietic rudiments, and neural tube closure (Graham, 2003; Bertrand et al., 2005; Wallingford, 2005); however, the underlying cellular and molecular mechanisms are poorly understood. Studies have shown that cell migration is highly regulated and involves the extension of leading processes, where continuous remodeling of actin and adhesive contacts is required (Li et al., 2005). Interestingly, we observed strong *NF2* promoter activity in sites where migrating cells were located including the neural tube closure, the branchial arches, the dorsal aorta, and the paraaortic splanchnopleura. The most intense activity was detected along the dorsal midline during neural tube closure, the location where the adhesion and fusion of two opposing neural folds and epithelial sheets occur. Similarly, high levels of *NF2* promoter activity were seen at the site of the anterior neuropore closure in the head region. Notably, McLaughlin et al. (2004) recently generated conditional *Nf2* knockout mice in which *Nf2* was deleted throughout the developing central nervous system by using nestin promoter-driving *Cre* recombinase. These mice displayed defects in neural tube closure and tissue fusion. It is known that cell adhesion during dorsal closure relies on the activities of the dynamic actin-based protrusions (Jacinto et al., 2002; Woolner et al., 2005). Since merlin localizes to membrane ruffles and adherens junctions (Gonzalez-Agosti et al., 1996; Shaw et al., 1998; Maeda et al., 1999) and plays critical roles in cell motility and cell adhesion (Gutmann et al., 1999; Stokowski and Cox, 2000; Lallemand et al., 2003), the most intense *NF2* promoter activity along the dorsal midline and at the site of anterior neuropore closure that we detected suggests that merlin may be necessary for cytoskeletal machinery driving cell adhesion and movement during neural tube closure.

In addition, it is tempting to speculate that merlin may participate in neural crest cell

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3 migration. The neural crest comprises a group of highly motile cells which are the precursors of  
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5 peripheral neurons, Schwann cells, pigment and facial cartilage cells (LeDouarin and Kalcheim,  
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7 1999; Jessen and Mirsky. 2005). Intriguingly, we detected little *NF2* promoter activity in  
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9 premigratory neural crest cells and the round-shaped neural crest cells, which had just  
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11 delaminated from the dorsal ridge region of the neural fold. On the contrary, significant *NF2*  
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13 promoter activity was found in the neural crest cells already migrating away from the dorsal  
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15 neural tube. Such a transient change of *NF2* promoter activity implies a transcriptional  
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17 regulation during neural crest cell migration and further corroborates with the role of merlin in  
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19 cell motility and cell adhesion (McClatchey, 2004; McClatchey and Giovannini, 2005). It is  
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21 possible that down-regulation of *NF2* promoter expression may allow premigratory neural crest  
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23 cells to delaminate from the dorsal neural tube. Once migrating away, the neural crest cells turn  
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25 on the *NF2* gene to ensure cell migration and cell adhesion in order to colonize different parts of  
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27 the embryo. Recently, several developmentally regulated transcription factors have been  
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29 implicated in the control of neural crest induction and delamination (Cheung et al., 2005). Thus,  
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31 it will be important to see whether these transcription factors regulate *NF2* promoter expression  
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33 during neural crest cell migration.  
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41 Analogously, the *NF2* promoter was highly expressed in hematopoietic stem cell-producing  
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43 tissues such as the yolk sac and the paraaortic mesenchyme. This mesodermally derived  
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45 intraembryonic region, known as the aorta-gonad-mesonephros region or, at a slightly earlier  
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47 developmental stage, the paraaortic splanchnopleura, produces, respectively, potent  
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49 hematopoietic stem cells and multipotent progenitor cells in addition to the yolk sac (Bertrand et  
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51 al., 2005). The strong *NF2* promoter activity in these hematopoietic stem cell-producing tissues  
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53 suggest that merlin may also play a role in the migration of hematopoietic progenitors into these  
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embryonic hematopoietic rudiments including fetal liver, thymus, spleen, and bone marrow during embryonic development.

As mentioned above, in addition to vestibular schwannomas, most NF2 patients develop multiple schwannomas that are associated with trigeminal nerve and spinal nerve roots, and less commonly, meningiomas and ependymomas (McClatchey, 2004; McClatchey and Giovannini, 2005; Neff et al., 2005). We detected significant *NF2* promoter activity in all these affected tissues during embryonic development. In particular, very intense promoter activity was noted in the tela choroidea, which consists of a layer of ependymal cells covered by the meninges. Significant *NF2* promoter activity was also seen in the acoustic ganglion, trigeminal ganglion, spinal ganglia, and their extending nerves. Furthermore, NF2 patients frequently suffer from juvenile lens opacities and a variety of retinal and optic nerve lesions including defects of the pigment epithelium and pigment epithelial retinal hamartomas (Evans et al., 1992; Parry et al., 1994; Meyers et al., 1995; Hazim et al., 1998; Levine and Slattery, 2003). Consistent with previous observation (Claudio et al., 1995; Huynh et al., 1996), we detected some *NF2* promoter activity in the lens. Strong promoter activity was also seen in the optic chiasma. In the retina, *NF2* promoter is highly expressed in the pigmented epithelium. The fact that the *NF2* promoter is very active in the tissues affected by NF2 during embryonic development further support the role of merlin in the pathogenesis of this genetic disorder.

It should be noted that the 2.4 kb *NF2* promoter appeared to be sufficient to recapitulate most of the endogenous *Nf2* RNA expression pattern in the embryonic tissues during embryogenesis, as we compared the  $\beta$ -gal staining pattern with the results from the RNA *in situ* hybridization (Figures 3, 4, and 5; McClatchey et al., 1997; McLaughlin et al., 2004). A detailed comparison with embryo sections from *in situ* hybridization analysis will strengthen this

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3 conclusion. However, while the 2.4-kb *NF2* promoter could direct  $\beta$ -gal expression to some  
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5 extraembryonic tissues such as allantois and yolk sac, no expression was detected in the  
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7 ectoplacental cone and chorionic ectoderm in transgenic embryos at E6.5~7.5, the time when *Nf2*  
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9 knockout mice show defects in extraembryonic tissues. We hypothesize that additional elements  
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11 located in the upstream or downstream region of the *NF2* promoter are required for proper  
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13 expression in these extraembryonic tissues.  
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18 Previously, we (Welling et al., 2000; Chang et al., 2002) showed that while multiple  
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20 elements are required for full *NF2* promoter activity in transfected cells, a GC-rich sequence,  
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22 which was located in the promoter proximal region and could be bound by transcription factor  
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24 Sp1, served as a positive *cis*-acting regulatory element. We are presently conducting  
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26 experiments to test whether the GC-rich sequence and other *cis*-acting regulatory elements are  
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28 important for the spatial and temporal expression pattern of the *NF2* promoter. Understanding of  
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30 the regulation of the *NF2* gene *in vivo* may provide us new clues regarding merlin's participation  
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32 in cell migration and cell adhesion during embryonic development.  
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## FIGURE LEGENDS

**Figure 1.** Schematic diagram of the pNF2P2.4-n $\beta$ gal construct and various mouse cDNA fragments used in whole-mount RNA *in situ* hybridization. (A) The pNF2P2.4-n $\beta$ gal construct contains the 2.4-kb human *NF2* promoter fused with a nuclear localization signal (nls)-containing  $\beta$ gal expression cassette. SS, SV40 splicing signal; An, SV40 polyadenylation sequence. (B) Various mouse *Nf2* cDNA fragments were obtained by RT-PCR as described in Materials and Methods and cloned into the pCRII-TOPO vector. The relative locations of the *Nf2* cDNA fragments are illustrated. (C) Nucleotide sequences and locations of the mouse *Nf2*-specific primers. \*Nucleotide position +1 is assigned to the A residue of the ATG translation start codon (GenBank accession No. [L27090](#)). <sup>+</sup>The ATG translation start codon is underlined. The primer sequence in the *Nf2* coding region is shown in bold letters while that in the 5' or 3' untranslated region is indicated in small letters.

**Figure 2.** Expression of  $\beta$ gal in transgenic E5.5~7.5 embryos. (A) Images of whole mount X-gal stained transgenic embryos at E5.5~7.5. eee, extraembryonic ectoderm; ee, embryonic ectoderm; m, mesoderm; e, endoderm; ec, ectoplacental cone; ce, chorionic ectoderm. Bar = 100  $\mu$ m. (B) Tissue section revealed strong  $\beta$ -gal staining in mitotic cells from embryonic ectoderm of the E6.5 embryo. Tissue section was photographed at 400X magnification. Arrows point to mitotic cells. Bar = 10  $\mu$ m.

**Figure 3.** Detection of endogenous *Nf2* RNA expression and  $\beta$ -gal staining in E7.5 embryos.

Whole-mount RNA *in situ* hybridization of wild-type E7.5 embryos was performed using an

antisense (A and C) or sense (B) *Nf2* 3U1 probe (Figure 1) derived from the exon 17 region as described in Materials and Methods. Compared to the results obtained from the sense probe control (B), strong *Nf2* RNA expression was detected in the embryo embedded in the decidua (A) or the dissected embryo (C), particularly in the developing neural ectoderm (arrow). Similarly, whole-mount X-gal staining showed strong  $\beta$ -gal expression in the developing neural ectoderm of the transgenic E7.5 embryo (D). The slight difference in the size and shape of the embryo shown in panels C and D was due to the procedures. The embryo processed for *in situ* hybridization was dehydrated with methanol, followed by proteinase K digestion and fixation. The embryo processed for  $\beta$ -gal was fixed in the fixation solution before X-gal staining. Nevertheless, the  $\beta$ -gal staining pattern in the embryonic tissue was similar to the endogenous *Nf2* RNA expression pattern.

**Figure 4.** The *Nf2* RNA expression and  $\beta$ -gal staining pattern in E8.5 embryos. (A-D) The pattern of strong *Nf2* RNA expression in the developing neural tube of the wild-type E8.5 embryo was confirmed by the  $\beta$ -gal staining of the E8.5 transgenic embryo. Whole-mount RNA *in situ* hybridization of wild-type E8.5 embryos was performed using an antisense (A) or sense (B) *Nf2* probe as described in Figure 3. Whole-mount X-gal staining was also performed on transgenic (C) or non-transgenic (D) E8.5 embryos. Note that the developing neural tube (arrow) showed strong *Nf2* RNA or  $\beta$ -gal expression. In addition, *Nf2* expression was also detected in the allantois (arrowhead). The small photograph insert in panels A and C displays the dissected embryo from *in situ* hybridization and  $\beta$ -gal staining analysis, respectively. (E-G) Change of *NF2* promoter activity during neural crest cell migration. (E) A transverse section of the transgenic E8.5 embryo showed significant  $\beta$ -gal expression in neural fold (nf) of the head

region, the developing neural tube (nt) and heart (h), as well as yolk sac (ys) and allantois (a). Note that the tip (arrows) of the neural fold displayed weak  $\beta$ -gal staining compared to the rest of the neural fold which exhibited strong  $\beta$ -gal activity. Bar = 100  $\mu$ m. (F-G) Detailed analysis of tissue sections containing the neural fold region revealed that while little  $\beta$ -gal staining was found in the round-shaped neural crest cells (arrows) which were at the moment of delaminating from the dorsal ridge region of the neural fold (F), significant  $\beta$ -gal expression was detected in the neural crest cells already migrating away from the dorsal neural tube (G). da, dorsal aorta.

**Figure 5.** Strong *Nf2* RNA expression and  $\beta$ -gal staining were detected in the developing brain, the branchial arches, and the paraaortic splanchnopleura of E9.5 embryos. *In situ* hybridization of wild-type E9.5 embryos was performed using an antisense (A) or sense (B) *Nf2* probe as described before. Whole-mount X-gal staining was also performed on transgenic E9.5 embryos (C). Sagittal section of the  $\beta$ -gal stained embryo was obtained and shown in panel (D). Arrows point to neural crest cell-populated branchial arches and the asterisk marks the location of the paraaortic splanchnopleura. fb, forebrain; mb, midbrain; hb, hindbrain; h, heart; da, dorsal aorta; ps, paraaortic splanchnopleura. Bar = 200  $\mu$ m.

**Figure 6. Immunohistochemical analysis of tissue sections from E9 (A-B) and E11.5 embryos (C-E).** Tissue sections were stained with anti-Sox9 (A,B,D), anti-S100 (C) and anti-merlin (E) antibodies according to Materials and Methods. A hematoxylin was used as a counterstain. The positively stained tissue appeared brown. Arrows point to migrating neural crest cells (A,B) or dorsal midline of the neural tube closure (D). nt, neural tube; sc; spinal cord, ag, acoustic ganglion; ov, otic vesicle; mt, metencephalon; tg, trigeminal ganglion.



**Figure 7.** Lateral views of whole-mount X-gal-stained transgenic mouse embryos at various days p.c. (A) E 9.5, (B) E10.5, (C) E12.5, and (D) E14.5. Bar = 400  $\mu$ m.

**Figure 8.** The most intense  $\beta$ -gal expression was detected along the dorsal closure (arrows) of neural tube in E9.5 (A) and E10.5 (B) transgenic embryos. Strong  $\beta$ -gal expression was also seen in the Branchial arches I-IV (arrows) of the E10.5 embryo (C).

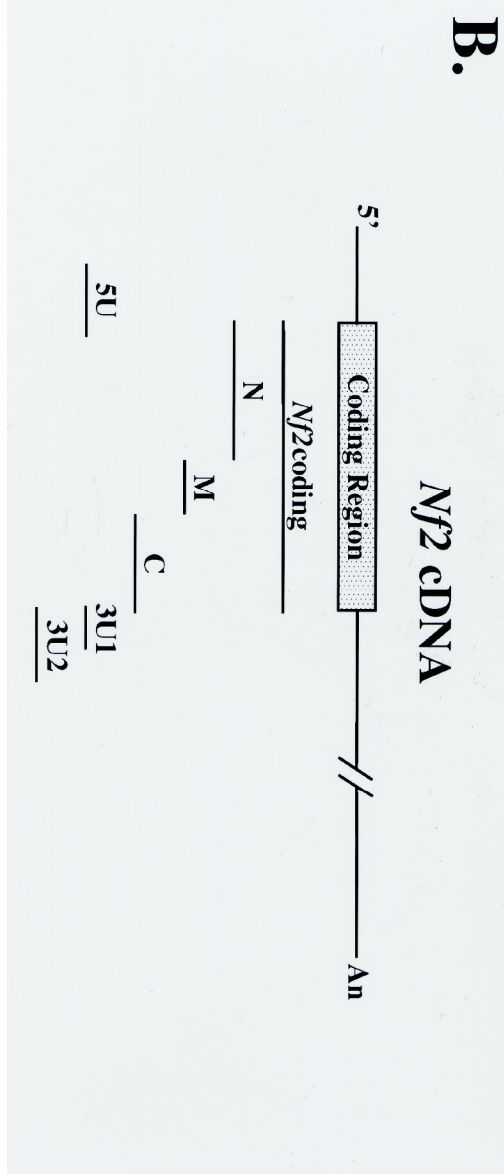
**Figure 9.** Strong  $\beta$ -gal staining in the neural ectoderm-derived tissues of E10.5 transgenic embryos. (A) Intense  $\beta$ -gal staining was detected in the metencephalon (mt), the tela choroidea (tc), and the myelencephalon (my). mc, myelocoel. Bar = 400  $\mu$ m. (B) Sagittal section of the head region revealed strong  $\beta$ -gal expression in the telencephalon (te), the diencephalon (de), the optic chiasma (oc), the tuberculum posterius (tp), and the infundibulum (i). Striped pattern of  $\beta$ -gal staining was seen in the mesencephalon (me), while little or no expression was detected in the Rathke's pocket (rp). Bar = 200  $\mu$ m. (B and D) Parasagittal sections. (C) Sagittal section of the eye showed that the prospective pigmented epithelium (pe) of retina displayed robust  $\beta$ -gal staining, while the neural layer (nl) of the retina and the lens (l) exhibited very little expression. Bar = 100  $\mu$ m. (D) Significant  $\beta$ -gal expression was detected in the mandible prominence of the first branchial arch (m), the paraaortic mesenchyme (pm), the atrium (a) and the ventricle (v) of the heart, and the dorsal aspect of the spinal cord (sc). Some  $\beta$ -gal expression was seen in the stomach region (s). Bar = 400  $\mu$ m.

**Figure 10.** Significant *NF2* promoter activity was detected in various *NF2*-affected tissues such

as the acoustic ganglion, the trigeminal ganglion, the spinal ganglia, and the pigmented epithelium of the retina in transgenic E11.5 embryos. (A, E, and F) Transverse sections. (B-D and G) Sagittal sections. (A) Strong  $\beta$ -gal expression was detected in the telencephalon (te) and the diencephalon (de). me, mesencephalon. Bar = 500  $\mu$ m. (B) The myelencephalon (my) showed a striped pattern of  $\beta$ -gal expression. The peripheral region of the acoustic ganglion (ag; cranial ganglion VIII) and its extending nerve also stained positive for  $\beta$ -gal expression. ov, otic vesicle. Bar = 250  $\mu$ m. (C) Intense  $\beta$ -gal expression was found in the pigmented epithelium (pe) of the retina. Also, strong  $\beta$ -gal expression was seen in the trigeminal ganglion (tg; cranial ganglion V) and its nerve branches. Bar = 250  $\mu$ m. (D) Robust  $\beta$ -gal staining continued to be seen along the dorsal midline (arrow) of the spinal cord (sc). Positive  $\beta$ -gal staining was also detected in the wall of the dorsal aorta (da). Bar = 200  $\mu$ m. (E) Strong  $\beta$ -gal expression was found in the dorsal aspect (arrow) of the spinal cord (sc). Positive  $\beta$ -gal staining was also detected along the dorsolateral and late ventral pathways of neural crest cell migration surrounding the spinal ganglion (sg). da, dorsal aorta. Bar = 300  $\mu$ m. (F) The sclerotome of somites showed strong  $\beta$ -gal expression. While the mesonephros (mn) exhibited positive  $\beta$ -gal staining, the gonad (g) showed little expression. Bar = 200  $\mu$ m. (G) Significant  $\beta$ -gal expression was observed in the paraaortic mesenchyme (pm), the heart, particularly the endocardium including the valves, the liver (l), and the mesonephros (mn). a, atrium; v, ventricle; pn, pronephros; s, stomach; g, gonad. Bar = 500  $\mu$ m.

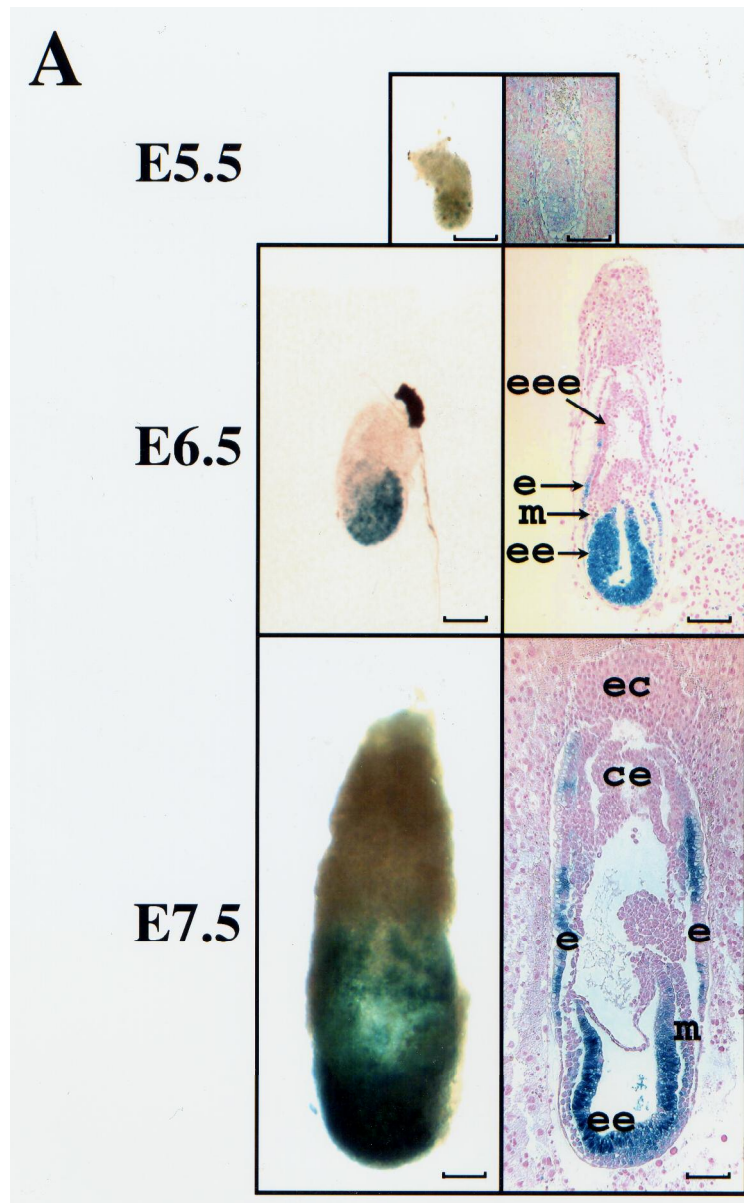
**Figure 11.** The  $\beta$ -gal staining pattern in various neural tissue sections from transgenic E12.5 embryos. Whole-mount X-gal stained embryos were prepared and sagittal sections were

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3 obtained as described before. (A) Intense  $\beta$ -gal expression was found in the telencephalon (te).  
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6 (B) Deep  $\beta$ -gal staining continued to be detected in the pigmented epithelium of the retina (pe).  
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9 di, diencephalon. (C) Strong  $\beta$ -gal staining was observed in the posterior commissure (pc)  
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11 compared to that in the mesencephalon (me), which showed patchy expression. (D) The tela  
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13 choroidea-derived posterior choroid plexus (pcp) in the 4<sup>th</sup> ventricle area was deeply labeled.  
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15 me, mesencephalon; i, isthmus; mt, metencephalon. (E) Only certain areas in the tuberculum  
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17 posterius (tp) were positive for  $\beta$ -gal staining. (F) Significant  $\beta$ -gal expression was also  
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19 observed in the trigeminal ganglion (tg) and its nerve divisions. mt, metencephalon. (G) Cells  
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21 surrounding the spinal ganglia (sg) and their extending nerves continued to show  $\beta$ -gal staining.  
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N/2 Primer	Sequence <sup>+</sup>	Position*
Coding-F	5'-ggtacccccgcgc <u>atgg</u> ccggaagccatcgct-3'	-13 ~ +18
Coding-R	5'-tcacccgtctagagttcttccaagaag-3'	+1798 ~ +1773
5U-F	5'-acaacgtgtgctgcgtctcccaactacttg-3'	-406 ~ -383
5U-R	5'-cgatccgcacacgttgaatgtcttgg-3'	+79 ~ +56
3U1-F	5'-ggccttggacggtccctacacacagcg-3'	+1668 ~ +1690
3U1-R	5'-cgggggcccctcactcaagagttcc-3'	+1913 ~ +1890
3U2-F	5'-cagagcgcccaagtcccgagtg-3'	+1773 ~ +1798
3U2-R	5'-gcccatcccaagaagcccaagtttg-3'	+2244 ~ +2223



**B**

